REDOX MECHANISMS OF SELENOCOMPOUNDS IN LUNG CANCER PREVENTION AND TREATMENT

by

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ABSTRACT

Lung cancer is the most fatal and second most prevalent type of cancer in the United States with a current five-year survival rate of only 16%. Thus, novel therapeutic agents to both prevent and treat lung cancer are necessary. One such agent is selenium, a micronutrient present in the diet. Epidemiological studies and supplementation trials with selenium have shown it to decrease lung cancer incidence and mortality. Selenium has also been shown to decrease lung tumor burden in animal studies, with the benefit being compound dependent. The mechanisms of action of selenium in cancer remain under investigation, but may relate to cellular redox status regulation. The hypothesis of this work is that distinct selenocompounds alter the cellular redox state of human lung cells through the Nrf2/antioxidant response element (ARE) pathway and the antioxidant selenoprotein thioredoxin reductase 1 (TR1). This hypothesis was tested using three specific aims:

1. Determine the redox effects of selenocompounds in A549 adenocarcinoma cells and BEAS-2B nonmalignant bronchial epithelial cells.
2. Investigate the ability of selenocompounds to activate the Nrf2/ARE pathway in nonmalignant BEAS-2B cells.
3. Determine if TR1 modulates the cytotoxcity of selenocompounds in malignant A549 cells.

Several selenocompounds were investigated, including the selenoamino acids selenomethionine and selenocystine, the selenocysteine prodrugs 2-butyl selenazolidine-4(R)-carboxylic acid (BSCA) and 2-cyclohexylselenazolidine-4(R)-carboxylic acid (ChSCA), and methylseleninic acid (MSA). This work indicates that selenium can modulate cellular redox status, but the effects are compound and cell-line specific. Selenocystine and ChSCA induced oxidative stress in A549 cells and activated the Nrf2 pathway in BEAS-2B cells. Selenocystine, ChSCA and BSCA also demonstrated enhanced cytotoxicity in A549 cells with TR1 knockdown, which was related to their ability to deplete intracellular glutathione. MSA produced a reductive stress in A549 cells and activated the Nrf2 pathway in BEAS-2B cells, but its cytotoxicity was not altered by TR1 status. Selenomethionine failed to modulate cellular redox status, activate the Nrf2 pathway, or demonstrate enhanced cytotoxicity with TR1 knockdown. These findings further demonstrate that selenium has compound-dependent redox effects and certain compounds, namely selenocystine and ChSCA, may have actions as both cancer preventive and anti-tumor agents in the lung.

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Aldh1a1	aldehyde dehydrogenase family 1 member A1
AIF	apoptosis inducing factor
ASK1	apoptosis signaling kinase 1
β2M	beta-2 microglobulin
BSCA	2-butylselenazolidine-4(R)-carboxylic acid
BSO	L-buthionine-(S,R)-sulfoximine
Ces1	carboxylesterase 1
CDDP	*cis*-platinum(II) diammine dichloride
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
CCK-8	Cell Counting Kit-8
ChSCA	2-cyclohexylselenazolidine-4(R)-carboxylic acid
Cys	cysteine
DCF	dichlorofluorescein
H_2DCFDA	2,7-dichlorofluroescein diacetate
DMSO	dimethylsulfoxide
ER	endoplasmic reticulum
EASE	Expression Analysis Systematic Explorer
GCLc	glutamate-cysteine ligase, catalytic subunit
GSH	glutathione

GPx	glutathione peroxidase
JC-1	5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolyl-carbocyanine iodide
KEAP1	Kelch-like ECH-associated protein 1
MSCA	2-methylselenazolidine-4-carboxylic acid
MSA	methylseleninic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mBBr	monobromobimane
NAC	*N*-acetyl-L-cysteine
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NSCLC	non-small cell lung cancer
NHBE	normal human bronchial epithelial cells
NPC	Nutritional Prevention of Cancer
Nrf2	nuclear factor erythroid 2-related factor 2
OSCA	2-oxoselenazolidine-4(R)-carboxylic acid
OPLAH	5-oxoprolinase
PEITC	phenethyl isothiocyanate
PhSCA	2-phenylselenazolidine-4-carboxylic acid
PhOSCA	2-(2’-hydroxy)phenylselenazolidine-4-carboxylic acid
p-XSC	1,4-phenylenebis(methylene)selenocyanate
PI	propidium iodide
ROS	reactive oxygen species
Se-MSC	selenium-methylselenocysteine
SeCys	selenocysteine

SECY	selenocystine
SELECT	Selenium and Vitamin E Cancer Prevention Trial
SEM	selenomethionine
SAM	Significance Analysis of Microarrays
Srxn1	sulfiredoxin 1
SF	sulforaphane
tet	tetracycline
Trx	thioredoxin
TR	thioredoxin reductase
Ugt1a6b	UDP glucuronosyltransferase 1a6b
UPR	unfolded protein response

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CHAPTER 1

RATIONALE FOR EXAMINING THE REDOX EFFECTS OF SELENIUM IN CANCER

Lung Cancer

Lung cancer is the most fatal and second most prevalent type of cancer in the United States [1]. Lung cancer is divided into two classes: small cell and non-small cell. Of the two, non-small cell lung cancer is the more prevalent, accounting for 85% of lung cancers [2]. Non-small cell lung cancer can be further subdivided into three types: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma, with adenocarcinoma being the most common. Adenocarcinomas develop in alveolar type II cells and typically occur in the periphery of the lung. Tobacco smoking is the number one risk factor for lung cancer development, accounting for over 85% of cases [2]. Other environmental risk factors include exposure to radon, asbestos and second-hand smoke. There is some evidence for cytochrome P450 1A1 and glutathione S-transferase M1 polymorphisms as genetic factors for increased susceptibility [3, 4], but these continue to be evaluated. Lung cancer rates in the United States are beginning to decline due to decreased tobacco smoking; however, lung cancer is expected to remain a public health problem for the next 50 years. Former smokers continue to be at higher risk for lung cancer than never smokes even decades after cessation [5]. Worldwide smoking rates and

lung cancer incidence continue to increase, especially in China [6].

The current long-term prognosis for most patients with lung cancer is poor. The five-year survival rate for lung cancer has only increased from 13% for patients diagnosed between 1975 and 1977 to 16% for diagnosis between 1996 and 2005 [1]. Poor clinical outcome is attributed to the typical late stage diagnosis of lung cancer and failure of current chemotherapy treatments. Almost 70% lung cancers are not diagnosed until they are locally advanced (Stage III) or metastatic (Stage IV) [6]. Once progressed to these late stages, lung cancer is not treatable by surgical resection. Lung cancer is also not treatable by resection in approximately 40% of patients with early stage disease due to co-morbidities [2].

First-line chemotherapy regimens for lung cancer generally include a platinum agent (cisplatin or carboplatin) in combination with an anti-mitotic agent (paclitaxel, docetaxel, or vinorelbine) or the topoisomerase I inhibitor irinotecan. The epidermal growth factor receptor (EGRF) tyrosine kinase inhibitors gefitinib and erlotinib have demonstrated efficacy in tumors with high EGFR expression, and monoclonal antibodies against vascular endothelial growth factor, such as bevacizumab, have also shown some success [6]. However, only a minority of patients responds and tumors often reoccur after resection and first-line chemotherapy. Second-line therapies are generally unsuccessful and only extend survival a few months [7]. Thus, there is a great need to develop agents that will prevent lung cancer development and progression, as well as improve treatment.

Few lung cancer chemoprevention trials have been conducted, but they generally have not been successful. The finding that lung cancer incidence is negatively correlated with a diet rich in fruits, vegetables, and vitamins [8] and serum antioxidant levels [9]

spurred the investigation of vitamins and antioxidants in clinical supplementation trials. The vitamin trials surprisingly showed an increase in lung cancer incidence and mortality with β-carotene and no effect of α-tocopherol or retinoids [10-12]. Cigarette smoke has since been shown to oxidize these vitamins in lung cells [13-15], indicating that they might exert prooxidant effects in the current smoker populations of these studies rather than function as antioxidants. The glutathione precursor *N*-acetylcysteine decreased DNA adduct formation in bronchoalveolar lavage fluid from smokers but demonstrated no benefit on overall survival or event-free survival in patients with lung cancer [16, 17].

Phytochemicals have also been of interest as chemoprevention agents. This is a structurally diverse group of non-nutritive plant components that are thought to be chemopreventive by inducing phase II enzymes. One class is the isothiocyanates, which are sulfur-containing compounds found in cruciferous vegetables such as broccoli. Sulforaphane is a methylsulfinylalkyl isothiocyanate that has been shown to induce phase II enzyme expression and activity in the lung both *in vitro* and *in vivo* [18]. Despite this efficacy in preclinical models, no evidence for phase II gene induction has been found following short term dietary intervention with broccoli [19], but pure sulforaphane has not been evaluated. Phenethyl isothiocyanate (PEITC), an aromatic isothiocyanate, is effective at inhibiting lung tumorigenesis in animals [20] and hypothetically inhibited the oxidative metabolism of tobacco carcinogens in smokers who consume watercress, a rich source of a PEITC precursor [20]. However, there is little data for PEITC inducing phase II enzymes in the lung. It is thought that this isothiocyanate works primarily through cytochrome P450 inhibition to prevent the generation of reactive tobacco carcinogen metabolites [20]. Therefore, PEITC might work best in current smokers, who would be

concurrently exposed to tobacco carcinogens, rather than in former smokers. Polyphenols and flavins from teas are effective in the post-initiation phase, but high doses are needed to provide a benefit due to presumed low bioavailability [21]. Green tea was also studied in humans for the treatment of lung cancer, but no benefit against existing tumors was found [22]. The synthetic dithiolethione oltipraz, a phase II enzyme inducer in preclinical models, failed to decrease DNA adduct formation, modulate phase II enzyme expression or activity, or increase glutathione in chronic smokers and appears to have toxicity issues that prevent its use as a chemopreventive agent [23].

Although studies have demonstrated mixed results of dietary compounds in preventing lung cancer at best, two studies have shown a benefit in patients with diagnosed lung cancer. High dose vitamin A adjuvant treatment of stage I lung cancer patients decreased new primary tumor formation and increased the disease-free interval [24]. Multivitamin use increased the survival time of small cell and non-small cell lung cancer patients [25, 26], but which vitamin produced the benefit or if it was a combination remains unknown.

Together these clinical and animal studies show that while an association between a plant-based diet and decreased cancer risk exists, the observed benefits in human trials are moderate at best. Additionally, nutritional supplements might have differential mechanisms in preventing lung cancer compared to treating established lung cancers. Further investigation of dietary compounds for lung cancer prevention, such as selenium, is necessary. The studies described in this work focus on using selenium as an agent whose unique redox mechanisms may make it a more efficacious dietary compound in lung cancer chemoprevention.

Selenium and Human Health

Selenium is a trace element discovered in 1817 by Jons Joakob Berzelius. It is a nonmetal and found in both organic and inorganic compounds. Selenium has the atomic number 34 and is chemically related to the other Group 16 element sulfur. It is found in soil and present in the diet as a micronutrient. Selenium was originally thought to be a carcinogen from the toxic effects observed in grazing cattle, but found to be essential for human health in 1957 in that it prevented liver necrosis [27].

The U.S. Recommended Dietary Allowance of selenium for adults is 55 μg, the level shown to be adequate for selenoprotein synthesis [28]. Brazil nuts are especially rich in selenium; other food sources include grains, cereals, seafood, and meat. The selenium content of plants is related to the selenium content of the soil in which they were grown, and meat selenium content comes from the plants and forage crops eaten by animals. Selenium from the diet can enter the body in the form of two selenoamino acids: selenocysteine and selenomethionine. Selenium can also be introduced as Se-methylselenocysteine from selenium concentrating plants such as garlic and broccoli. In mammals, selenocysteine present in proteins is the predominant biological form of selenium even though selenomethionine is present in the diet at a greater amount than selenocysteine. This is because selenocysteine insertion, rather than selenomethionine, is specified in selenoproteins. The relationship between selenium plasma levels and all-cause mortality follows a U-shaped curve, with detrimental effects at both the low (<90 ng/mL) and high (>150 ng/mL) ends of plasma selenium concentration [29]. The ideal selenium plasma level is approximately 130 ng/mL [29]. In the United States, individuals

are generally selenium adequate and consume selenium at greater amounts than the recommended 55 μg/day [29].

Selenium deficiency is implicated in the development of several diseases [30]. Keshan disease is a cardiomyopathy named for the county in China where it was first observed. Dietary levels of selenium are low (<19 μg per day) in this and other regions of China due to low soil selenium levels. Selenium supplementation is able to ameliorate the cardiac disease and pulmonary edema associated with Keshan. Another disease associated with selenium deficiency is Kashin-Beck, an osteoarticular condition that affects joint cartilage and results in bone deformities. Cases of Kashin-Beck syndrome have been reported primarily in China and northern Russia, another area with low selenium soil content. Debate over what constitutes selenium deficiency continues, as New Zealanders also have lower serum selenium levels that other populations, but no relation to disease incidences has been observed [31]. There is also a body of evidence for the involvement of Se deficiency in cancer susceptibility. As the focus of this work is on the role of selenium in cancer, this association between selenium status and cancer will be discussed in depth later in this chapter.

On the other side of the spectrum, excess selenium can also produce negative health effects. The maximum amount of selenium that has been deemed safe for lifetime daily intake is 350 μg per day [32]. Levels above 900 μg are toxic [30]. These amounts represent general guidelines, as they are not selenocompound specific. Chronic selenium toxicity results in a condition termed selenosis. Symptoms of selenosis include brittle hair and nails, diarrhea, dermatitis, nervous system abnormalities, lowered hemoglobin levels, a garlic odor on the breath, nausea, and fatigue [33] Observable changes in fingernails as

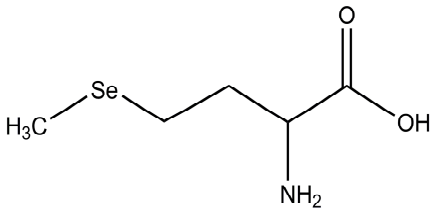
an indicator of selenosis begin to occur at 1600 μg per day [30]. Selenium toxicity can also occur from acute poisoning. A case report of acute selenium toxicity describes the ingestion of 10 g sodium selenite by a 75-year-old male for its reputed health benefits, resulting in hypotension, acidosis, hypokalemia, and ultimately cardiac arrest [34]. The toxicity of selenium is thought to stem from incorporation of selenium in place of sulfur in enzymes involved in cellular respiration and the generation of reactive species [34]. These toxic effects demonstrate that although a nutrient, selenium is not inherently benign.

Selenocompounds and Their Metabolism

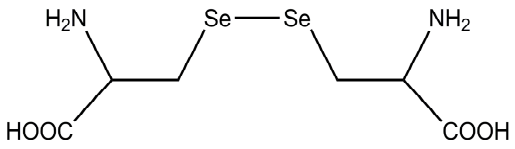
Distinct selenocompounds enter the selenium cellular metabolism pathway through either reduction or methylation, producing compound-specific metabolites (for selenocompound structures see Figure 1.1; for metabolism scheme see Figure 1.2). Depending on the dose and form, selenocompounds can be chemopreventive, toxic, or even carcinogenic [35]. The metabolism of a selenocompound may be a contributing factor in the observed differential efficacy of selenocompounds in chemoprevention.

The reductive metabolism pathway is responsible for generating hydrogen selenide [36]. Hydrogen selenide is thought to be the central selenium pool, where selenium can be converted into selenocysteine for insertion into selenoproteins or form methylated metabolites termed methylselenols. Sodium selenite, an inorganic selenocompound, is reduced to selenodiglutathione and then to hydrogen selenide by glutathione. Selenite is one of the more toxic forms of selenium. Its toxicity is thought to partially stem from this depletion of glutathione through its metabolism.

Selenoamino acids

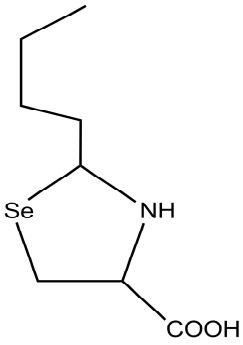


Selenomethionine
(SEM)

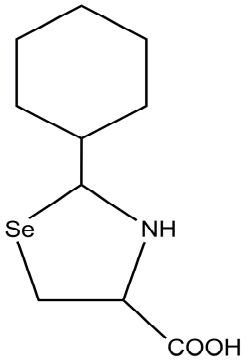


Selenocystine
(SECY)

Selenazolidines



2-butyl
selenazolidine-4-
carboxylic acid
(BSCA)



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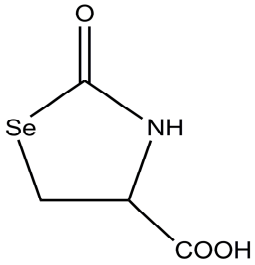
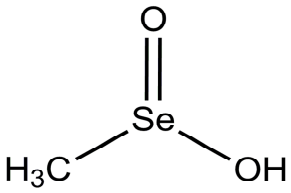
selenazolidine-4-
carboxylic acid
(ChSCA)

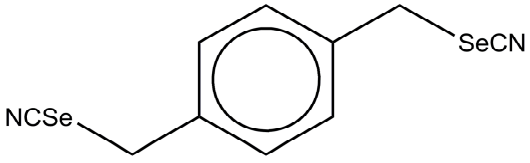


2-oxo
selenazolidine-4-
carboxylic acid
(OSCA)

Organoselenocompounds



Methylseleninic acid
(MSA)



1,4-phenylenebis(methylene)selenocyanate
(*p*-XSC)

Figure 1.1. Structures of organoselenium compounds of interest.

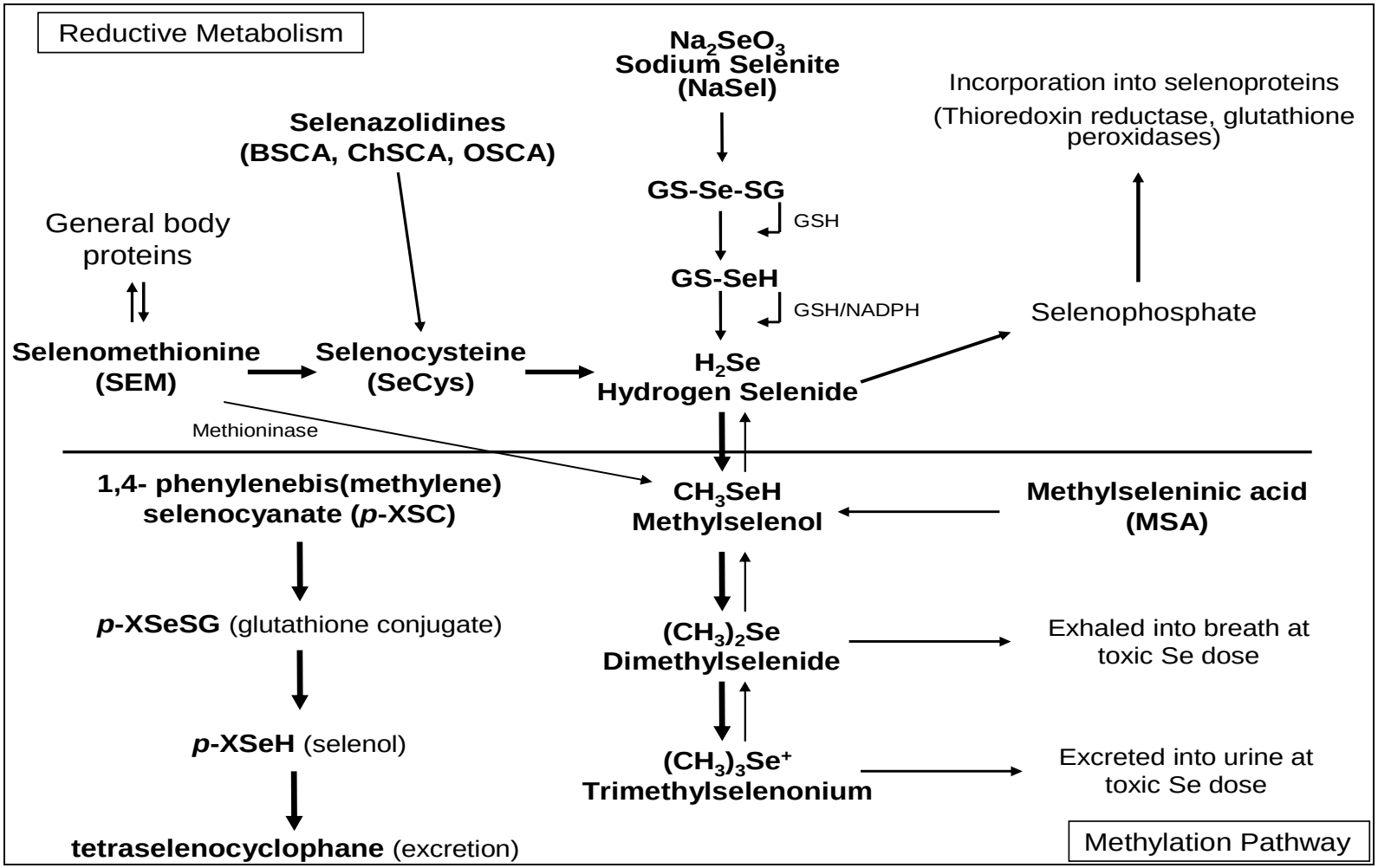


Figure 1.2. Entry of selenocompounds into the Se cellular metabolism pathway. Depending on the form, selenocompounds can enter cellular selenium pools through reductive metabolism to generate hydrogen selenide or via the methylation pathway to form methylselenol in the case of MSA or a selenol derivative in the case of *p*-XSC. Figure adapted from [36].

The selenoamino acids are also a part of the reductive pathway [36]. To form hydrogen selenide, selenocysteine first undergoes a glutathione-independent β-lyase reaction to cleave elemental selenium, which is then spontaneously reduced to hydrogen selenide [37]. Selenomethionine enters the hydrogen selenide pool by forming selenocysteine via the trans-sulfuration pathway [37]. Once in the hydrogen selenide pool, selenium can form selenophosphate by selenophosphate synthetase. Selenocysteine is synthesized from selenophosphate to be utilized in selenoprotein synthesis. While the selenoamino acids selenocysteine and selenomethionine both form the same hydrogen selenide intermediate, selenoproteins specifically incorporate selenocysteine. This is because selenoproteins have selenocysteine encoded for by a UGA codon upstream of a selenocysteine insertion sequence element [38]. For this reason, selenocysteine is referred to as the 21st amino acid [39]. Selenomethionine can leave the central selenium pool through nonspecific incorporation into general body proteins, as cells do not differentiate selenomethionine from methionine.

Also in the reductive metabolism pathway are the selenazolidines, a novel class of selenocompounds. Selenazolidines are designed to function as selenocysteine prodrugs. Selenocysteine itself is unstable, difficult to work with, and can oxidize to form the diselenide selenocystine; it is thought that these negative properties can be overcome through a prodrug approach. The selenazolidines were patterned after the thiazolidine class of sulfur compounds to serve as selenocysteine delivery agents with low toxicities [40]. Several different selenazolidines that have the same selenazolidine carboxylic acid ring structure, but differ in the 2-position functional group, have been synthesized. Depending on the functional group, these compounds are hypothesized to release

selenocysteine either enzymatically or nonenyzmatically. 2-oxo-selenazolidine-4-carboxylic acid (OSCA) was designed to release selenocysteine through a 5-oxoprolinase enzymatic reaction (Figure 1.3). The remainder of the synthesized selenazolidines, 2-butyl (BSCA), 2-cyclohexyl (ChSCA), 2-phenyl (PhSCA), 2-methyl (MSCA), and 2-(2'-hydroxy)phenyl (PhOHSCA) selenazolidine-4-carboxylic acid, are thought to release selenocysteine through nonenzymatic ring opening and hydrolysis (Figure 1.3). These selenazolidines that release selenocysteine through spontaneous hydrolysis also generate an aldehyde species. In addition to liberating selenocysteine, selenazolidines may also generate the selenocysteine dimer selenocystine. The selenazolidines also differ in their lipid partition coefficients, with OSCA and ChSCA being the most lipophilic and PhOSCA and MSCA being the least lipophilic [41].

Selenocompounds can form methylselenol independently of hydrogen selenide in what is termed the methylation pathway [36]. Methylseleninic acid (MSA) can directly form methylselenol through a nonenzymatic reaction. MSA is a synthetic selenocompound designed as Se-methylselenocysteine without the amino acid moiety. MSA does not require metabolism by β-lyase to produce methylselenol, while Se-methylselenocysteine does. MSA was designed to be a more efficacious form of Se-methylselenocysteine, since it can produce methylselenol in cells lacking β-lyase. The organoselenium compound 1,4-phenylenebis(methylene)selenocyanate (*p*-XSC) is thought to behave similarly to methylselenol in cells. *p*-XSC is hypothesized to form a glutathione conjugate (*p*-XSeSG), then undergo metabolism to selenol (*p*-XSeH), and finally be excreted as tetraselenocyclophane [42]. The possibility for the reductive metabolism of *p*-XSC and its entry into the hydrogen selenide pool has been indicated by

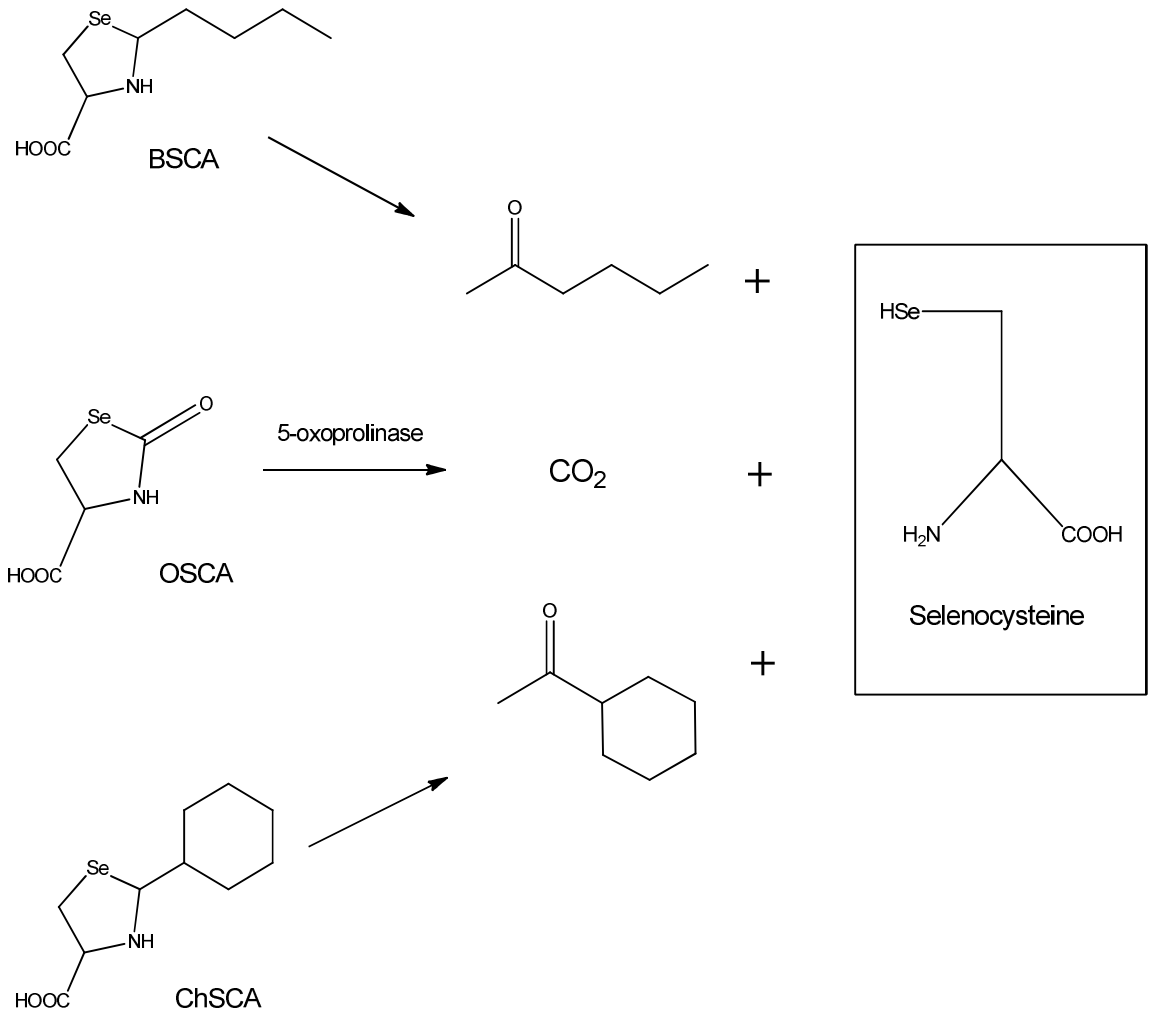


Figure 1.3. Formation of selenocysteine from the selenazolidines BSCA, OSCA, and ChSCA. BSCA and ChSCA are hypothesized to spontaneously release selenocysteine through hydrolysis, also generating aldehydes. OSCA is thought to release selenocysteine and CO_2 through a 5-oxoprolinase enzymatic reaction. Figured adapted from [40].

the ability of *p*-XSC to restore glutathione peroxidase activity in selenium-deficient rats, thereby contributing selenium to the hydrogen selenide pool for incorporation into selenoproteins [43]. However, this reductive metabolism of *p*-XSC is thought to be a minor metabolism pathway, as very low levels of the dimethyl selenide metabolite of hydrogen selenide were detected [42] and *p*-XSC was not incorporated into glutathione peroxidase as efficiently as selenite [43], which is primarily metabolized to hydrogen selenide. Selenomethionine can also directly form methylselenol by a methioninase reaction [44]. Hydrogen selenide can also be converted to methylselenol by methyltransferase [45], and methylselenol into hydrogen selenide via demethylation by demethylase.

The excretory metabolites of selenium are primarily methylated forms. In humans and rodents, the predominant mechanism for ridding the body of absorbed selenium is urinary excretion of methylselenosugars [46, 47]. Methylselenol can also form dimethylselenol and trimethylselenol through thiol S-methyltransferase reactions [45; 48]. Formation of these further methylated metabolites generally occurs only when selenium levels are very high and selenosugar synthesis is saturated. The di- and trimethyselenols are excreted through breath and urine, respectively. Fecal selenium excretion also occurs, but is primarily the route for unabsorbed selenium [49].

Selenium and Cancer

In 1969, Shamberger and Frost brought to attention the possible ability of selenium to protect against cancer in humans by citing two published studies [50]. One study by Allaway *et al*. was the first to associate selenium blood levels with cancer death

rates, finding a negative Pearson correlation coefficient of r = -0.96 [51]. The second study, by Kubota *et al*., found a lower death rate for regions of the United States with high forage crop selenium concentrations compared to those with low selenium concentrations [52]. An unpublished study showing a lower cancer death rate for Canadian provinces with selenium accumulator plants than provinces with plants that did not accumulate selenium was also described [50], further implicating selenium soil levels in mortality rates. Several other studies have confirmed these inverse associations between selenium plasma and soil levels and cancer mortality [53-56].

Since these original observations, inorganic and organic selenium compounds have been shown to decrease tumor formation in a number of chemical and viral carcinogenesis models [57]. It is important to note that the antitumor effects of selenium occur at supranutritional selenium levels, that is, above the levels needed to maximize selenoprotein activity [58]. The actions of selenium are both compound and model specific. An example is Se-methylselenocysteine, which is one of the most efficacious selenocompounds at reducing mammary tumors [59]. Se-methylselenocysteine is not effective at reducing lung tumors, whereas other selenocompounds have been [60]. Tissue distribution and expression of metabolizing enzymes such as β-lyase might explain the differential activity of selenocompounds in animal models.

In addition to numerous animal studies, epidemiological and case-control studies have also indicated a protective benefit of selenium in several cancers [57]. Some of the first clinical trials to test the ability of selenium prevent cancer in humans were conducted in Linxian, China. The population of this region is deficient in many nutrients, including selenium, and has high mortality rates for esophageal and gastric cancers. A combination

of selenium with β-carotene and α-tocopherol decreased total cancer mortality, stomach cancer mortality, and stomach cancer incidence [61], but did not decrease the prevalence of precancerous legions or early cancers in the esophagus or stomach in a smaller subset of participants evaluated by endoscopy [62]. To better evaluate the role of selenium in cancer prevention, two clinical trials were conducted in the United States: the Nutritional Prevention of Cancer (NPC) trial and Selenium and Vitamin E in Cancer Prevention Trial (SELECT).

The NPC trial was the first to evaluate selenium in cancer prevention [63]. It enrolled patients with a history of nonmelanoma skin cancers residing in the southeast United States, a recognized low soil selenium area. Selenium was supplied in the form of selenized yeast at 200 μg/day. No benefit of selenium was shown for preventing the reoccurrence of basal or squamous cell carcinoma in the initial follow-up time (4.5 mean treatment years), but decreases in prostate, colon and lung cancer incidence were found, as well as decreased rates of lung cancer and total cancer mortality. The reduction in prostate cancer incidence was the most statistically significant and was the only cancer for which the reduction remained significant in long-term follow up (7.9 mean treatment years) [64]. Further analysis of the NPC trial also showed that selenium was the most protective against prostate cancer in men with low baseline selenium levels [64-66]. Extended follow-up also indicated a significant increased risk of nonmelanoma skin cancers in participants with the highest baseline plasma selenium levels [66]. Overall, the findings of the NPC trial indicate that selenized yeast is efficacious in decreasing cancer mortality and preventing specific cancers, namely prostate, but baseline selenium status may be an important factor in this protective effect.

To further verify the NPC prostate cancer findings, The Selenium and Vitamin E in Cancer Prevention Trial (SELECT) began in 2001 and was designed with prostate cancer as its primary outcome [67]. This trial enrolled over 35,000 cancer-free men and provided oral 200 μg/day selenomethionine, 400 IU/day synthetic α-tocopherol, or both. The trial was scheduled to continue follow-up through 2013 but was stopped in October 2008 when analysis showed no decrease in prostate cancer incidence with either agent alone or in combination and no possibility that a benefit could occur in the follow-up period. No change in incidence of lung cancer, colon cancer, or all other cancers was found and no effect on cancer mortality shown. A statistically nonsignificant increase in type II diabetes was shown with selenium alone, a finding that was also reported for the NPC trial population within the highest baseline plasma selenium tertile [68].

Two hypotheses have been made to explain the conflicting results of these two selenium supplementation trials. First, the choice of selenocompound was different. The NPC trial used selenized yeast, which is comprised of greater than 65% selenomethionine [69, 70]. Selenocysteine, Se-methylselenocysteine, and other unknown organoselenium compounds account for the remaining total selenium content [70]. SELECT used pure selenomethionine since it is the predominant selenocompound in selenized yeast. It is possible that the other selenocompounds in selenized yeast that were not given in SELECT could be responsible for some benefit. Also, selenomethionine may not be the best form of selenium for producing biologically available selenium, as it can be non-specifically incorporated into general body proteins in place of methionine. Second, the baseline plasma selenium levels of the two study populations differed. The NPC trial enrolled subjects from the historically selenium low region of the southeastern United

States. This is in contrast with SELECT, where the majority of the population was selenium sufficient. Only 22% of men in SELECT were within the NPC trial's lowest two tertiles of plasma selenium levels. These results of the NPC trial indicated that low plasma selenium populations might benefit the most from selenium supplementation. Perhaps this low selenium population was too small in SELECT to demonstrate a benefit great enough to be seen when analyzing the entire population. To date, the data for the low selenium population in SELECT have not been analyzed to determine if a benefit occurred in this subpopulation. Despite the differential findings between the NPC trial and SELECT, it is important to note that they do not discredit the use of selenium in cancer prevention. Rather, they highlight the need for a better understanding of selenium biology and which populations might best benefit from supplementation [71, 72].

Proposed Mechanisms of Selenium in Cancer

Selenium is thought to function as a chemoprevention agent, defined as that which inhibits, reverses, or retards tumorigenesis [73], at multiple stages of neoplastic transformation. These stages include initiation, a rapid process when a cell is first exposed to carcinogens and DNA modifications occur; promotion, a lengthier process when proliferating preneoplastic cells accumulate; and progression, when a tumor with invasive and metastatic potential forms [73]. A range of mechanisms have been hypothesized for selenium in chemoprevention, including protection against oxidative stress, altered carcinogen metabolism, detoxification of carcinogenic intermediates, induction of apoptosis, cell cycle effects, immune system modulation, and inhibition of angiogenesis [74]. This work is focused on redox effects of selenium in the lung, with

studies in subsequent chapters relating to the ability of selenium to attenuate reactive species in nonmalignant cells and enhance apoptosis in malignant cells.

Selenocompounds have demonstrated capabilities *in vitro* that are attractive from a cancer therapy standpoint, that is, properties that would promote the death of a cell that has entered the transformation pathway and may become tumorigenic. Certain selenocompounds, such as selenite, selenocystine, MSA, and *p*-XSC can redox cycle with thiols, such as reduced glutathione, at physiological pH [44, 75, 76]. Selenomethionine does not produce superoxide in the presence of glutathione unless it generates methylselenol via the methionase reaction, which may explain its lower toxicity than other selenocompounds that can produce greater amounts of superoxide [44, 76]. These specific selenocompounds can redox cycle because they are able to form the RSe^- selenide anion (Figure 1.4). Redox cycling results in depletion of glutathione and generation of reactive species, such as superoxide, that can produce DNA strand breaks [36, 77]. This ability of selenocompounds to generate oxidative stress and DNA strand breaks has been directly associated with their ability to also induce apoptosis [78-80]. Certain selenocompounds have also been shown disrupt mitochondrial integrity by a mechanism involving thiol oxidation and superoxide generation [81, 82]. Through these mechanisms, selenium can promote cancer cell death through both caspase dependent and independent pathways, as demonstrated in several malignant cell lines [36, 83-87].

In regard to chemoprevention, two specific hypotheses for how selenium exerts its associated mechanisms exist. One hypothesis implicates increased formation of the selenium metabolite methylselenol as the basis for chemopreventive effects. Another hypothesis looks to increased selenoprotein synthesis.

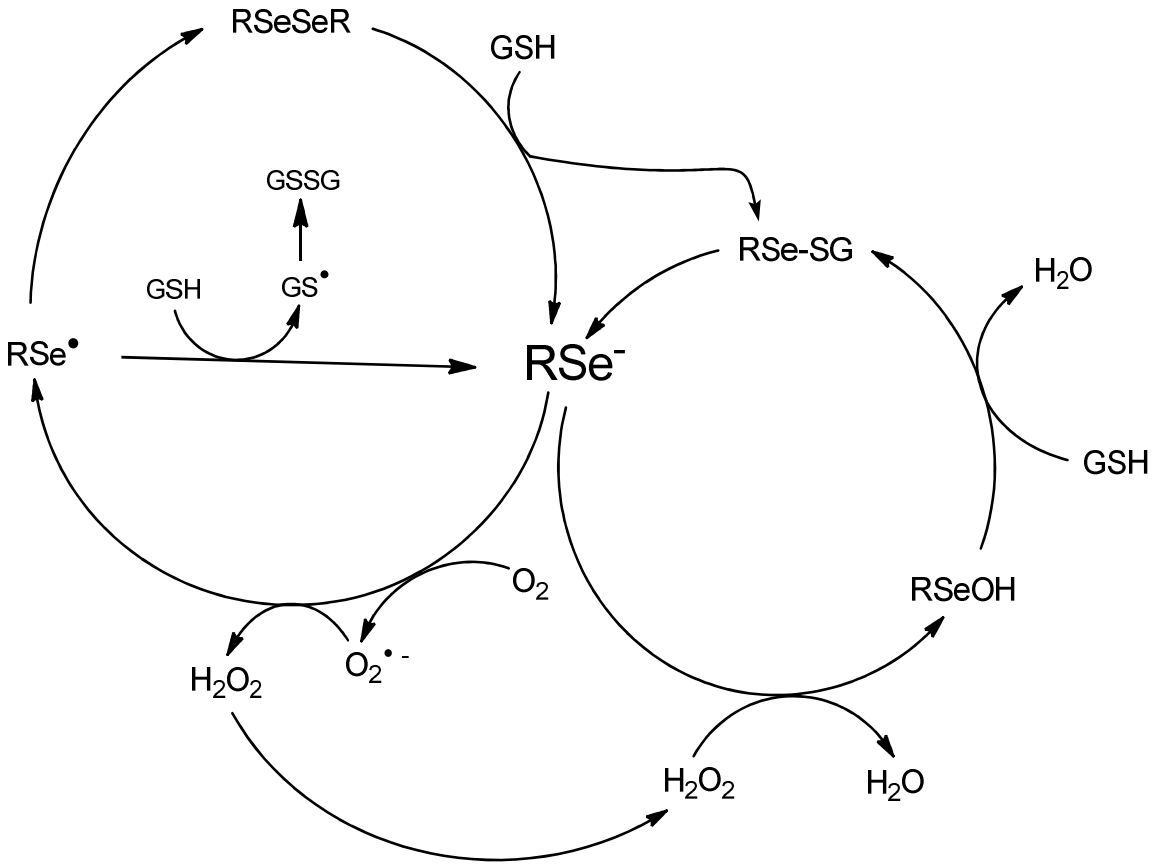


Figure 1.4. Redox cycling of selenides. Selenocompounds such as selenocystine, MSA, *p*-XSC, and selenite can form the selenide anion (RSe^-) and redox cycle with reduced glutathione (GSH), generating superoxide ($O_2^{\bullet -}$). Figure from [44].

Alkylselenols

Alkylselenols are low-molecular weight selenocompounds that can form by methylation of hydrogen selenide from the central selenium pool, or directly from such compounds as Se-methylselenocysteine and MSA. The alkylselenol class includes monomethylselenol, a key metabolite generated by Se-methylselenocysteine and MSA. Both Se-methylselenocysteine and MSA were found to be more effective than selenomethionine or selenite in a human prostate cancer xenograft model [88]. Selenized garlic, containing selenium primarily as Se-methylselenocysteine and its gamma glutamyl conjugate [69], was more efficacious in a mammary cancer model than selenite [89] or selenized yeast, which contained selenium primarily as selenomethionine [69, 89]. The superior efficacy of Se-methylselenocysteine and MSA in these studies is attributed to their ability to more readily form methylselenol than the other evaluated selenocompounds. Methylselenol precursors can inhibit cell cycle progression [90-92], induce caspase-mediated apoptosis [90] and prevent angiogenesis [93]. The methylselenol metabolite has been shown *in vitro* and *in vivo* to be responsible for the chemopreventive actions associated with Se-methylselenocysteine and MSA [94]. The exact mechanism by which methylselenols produce this benefit that other selenocompounds appear to lack is currently unknown.

Selenoproteins

To date, over 25 mammalian selenoproteins have been identified [95]. These proteins specifically contain selenium in their active sites in the form of genetically encoded selenocysteine. Proteins with nonspecific incorporation of selenium or selenium-

binding proteins are not grouped within this selenoprotein category. The most studied selenoproteins are the thioredoxin reductases and glutathione peroxidases, discussed in depth further in this chapter; the selenium transporter selenoprotein P; and the thyroid hormone activating iodothyronine deiodinases. Many of the identified selenoproteins have unknown functions that are just beginning to be elucidated. Selenocysteine is vital for the enzymatic activity of selenoproteins, as selenoproteins with mutated selenocysteine residues demonstrate significantly decreased activity [96]. Selenoprotein synthesis utilizes specific protein synthesis machinery to insert selenocysteine [95]. Insertion of selenium as selenocysteine is coded for by the UGA codon. In nonselenoproteins, UGA is a stop codon that terminates translation. For the UGA codon to be instead translated as selenocysteine, a selenocysteine insertion sequence (SECIS) element must be present downstream. This SECIS element is a stem-loop structure found in the 3'-untranslated region of eukaryotic selenoprotein mRNAs. To produce a selenocysteine-specific tRNA ($tRNA^{Sec}$), serine is first conjugated and then modified to phosphoserine. Selenium from the hydrogen selenide pool is phosphorylated to selenophosphate by selenophosphate synthetase 2 and then added to phosphoserine by selenocysteine synthetase. A $tRNA^{Sec}$-specific elongation factor and binding of the SECIS-binding protein 2 to the SECIS element are also required for selenocysteine incorporation [97]. Other currently unknown machinery may also be required. Under conditions of selenium deficiency, decreased selenoprotein synthesis and activity is observed [98].

One model for investigating the role of selenoproteins in cancer is the i^6A^- transgenic mouse. The $tRNA^{Sec,}$ is mutated *in vitro* by replacing an adenosine with a

guanosine, preventing the incorporation of selenocysteine into selenoproteins [99]. These mice have decreased selenoprotein expression, but do not exhibit an overt phenotype [99]. This strategy is employed to decrease selenoprotein expression rather than a $tRNA^{Sec}$ knockout mouse, as this is embryonic lethal [100]. In the azoxymethane model of colon cancer, the i^6A^- mouse was found to have more aberrant crypt foci than wild-type [101]. Selenium supplementation as selenite at supranutritional levels was able to non-significantly decreased the aberrant crypt foci in the i^6A^- mouse, indicating both selenoprotein dependent and independent mechanisms for chemoprevention by selenium. Selenoprotein deficiency was also found to increase lesions associated with prostate cancer in a transgenic cancer model [102]. One limitation of the i^6A^- mouse model is that it does not identify which selenoproteins are required for the observed benefit.

The relationship between selenoprotein expression and cancer risk has also been studied in humans. Decreased expression of selenoprotein P has been found in tumor tissues relative to matched nonmalignant tissue [103-105] and has also been correlated with risk of colon cancer [106]. Single nucleotide polymorphisms in the selenoprotein P gene have also been related to cancer risk [107]. As the primary function of selenoprotein P is to transport selenium to extrahepatic tissues, its decreased expression or activity could limit the amount of selenium available for selenoprotein synthesis or methylselenol formation [108]. Selenoprotein P may also have an antioxidant function that could contribute to decreased cancer risk [108, 109]. Glutathione peroxidase 1 (GPx1) is another selenoprotein that has been associated with cancer risk in humans. As a glutathione peroxidase, GPx1 primarily has an antioxidant function by reducing cellular hydroperoxides. Polymorphisms in GPx1 [110, 111] and allelic loss of GPx1 [112] are

associated with the development of several cancers. Polymorphisms and decreased expression of selenoprotein 15, which is located in the endoplasmic reticulum and functions in protein folding, have also been implicated [113]. While these studies indicate associations between certain selenoproteins and cancer, it is not known if changes in expression or activity are early occurrences that contribute to cancer development or occur later as a result of transformation.

One problem with the selenoprotein hypothesis as the single mechanism of selenium is that observed benefits occur at supranutritional levels [58]. This indicates that the associated benefits are not the result of increased selenoprotein expression or activity alone, as these are maximized at levels below supranutritional status. As the animal studies and clinical data indicate, maximal selenoprotein activity in conjunction with increased methylselenol production is the likely mechanism of chemoprevention [101].

Maintenance of Cellular Redox Status by Selenium

Selenium is thought to indirectly act as an antioxidant through the functions of selenoenzymes. This is in contrast to antioxidants that function by non-enzymatically reacting with oxidative species, such as α-tocopherol and ascorbic acid [114]. Control of redox status is important in chemoprevention, as oxidative stress can lead to DNA, protein, or lipid damage [115, 116]. Damage to these cellular components typically occurs in the initiation phase of carcinogenesis, ultimately resulting in mutagenesis [115]. Also, many of the signal transduction cascades for cell growth and proliferation, processes that become deregulated in cancer, are redox controlled [116]. The ability of

selenium to modulate redox status seems to be primarily related to the actions of two classes of selenoenzymes: the thioredoxin reductases and the glutathione peroxidases.

Thioredoxin Reductase

The main function of thioredoxin reductase is to reduce the redox-active disulfide in the thioredoxin active site [117, 118]. This reaction is carried out using electrons from NADPH cycled through flavin adenine dinucleotide (Figure 1.5). After the glutathione system, the thioredoxin system is the most important cellular antioxidant system. In humans, there are three thioredoxin reductase isoforms. Thioredoxin reductase 1 (TR1) is cytosolic, thioredoxin reductase 2 is mitochondrial, and thioredoxin reductase 3 is localized to the testes for involvement in spermatogenesis [117]. This work is focused specifically on the cytosolic TR1 isoform.

The TR1 isoform is expressed in all cell types and tissues, but in low concentrations relative to other antioxidants such as glutathione. Selenium supplementation has been shown to increase thioredoxin reductase activity until it plateaus [119]. In addition to thioredoxin, thioredoxin reductase can control the redox status of other substrates, such as thioredoxin-dependent peroxiredoxins and the tumor suppressor p53. An important role of the thioredoxin system is its function in deoxyribonucleotide biosynthesis. Thioredoxin provides electrons to ribonucleotide reductase for the conversion of ribonucleotides to deoxyribonucleotides, allowing for DNA synthesis in the S-phase of the cell cycle [120]. It is thought that this function leads to the requirement of TR1 and thioredoxin for embryogenesis [121, 122]. Thioredoxin reductase also reduces transcription factors, such as NF-κB and AP-1, thereby regulating

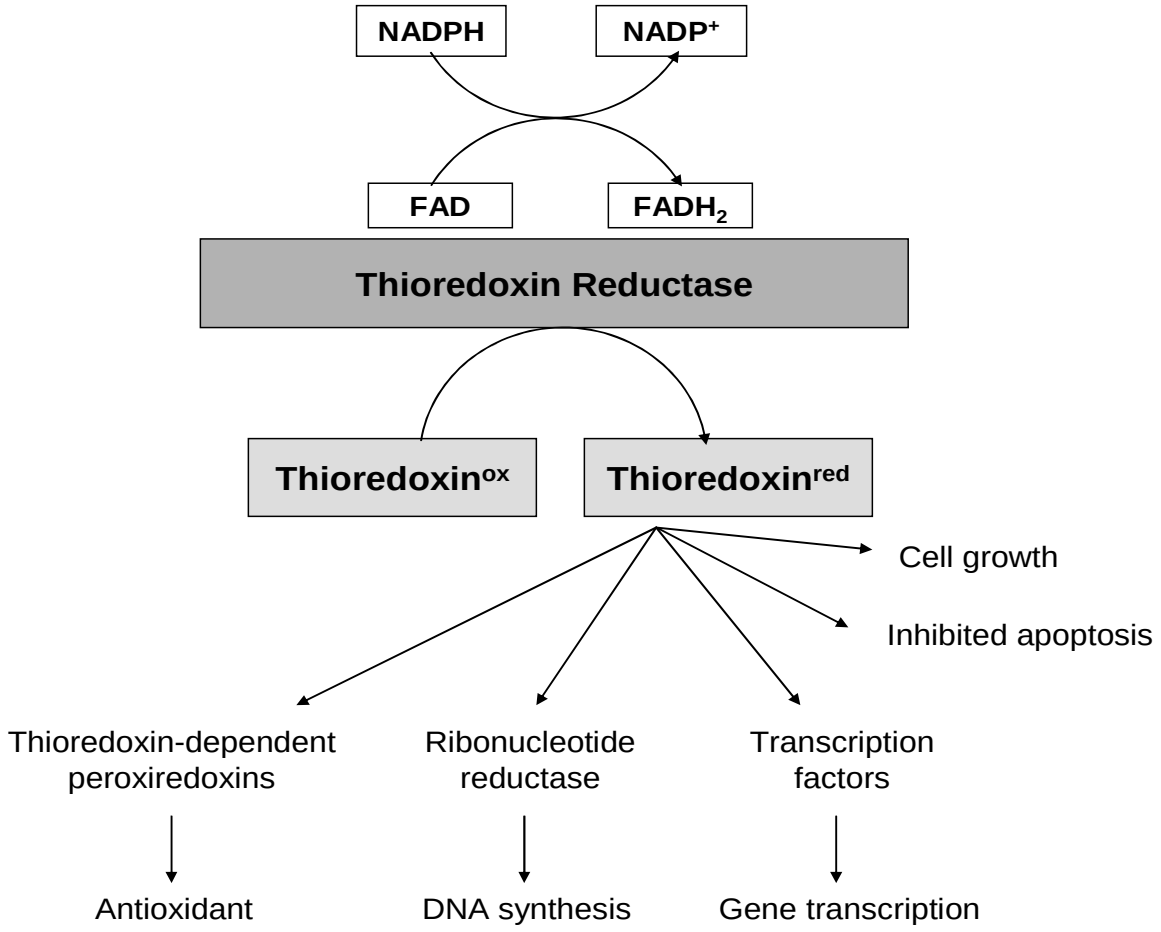


Figure 1.5. Maintenance of cellular redox status by the thioredoxin system. Thioredoxin reductase reduces oxidized thioredoxin by using electrons from NADPH cycled through flavin adenine dinucleotide (FAD). Thioredoxin can then maintain cellular proteins in a reduced state. The thioredoxin system is implicated in transcription factor activation, DNA synthesis for cell proliferation, and apoptotic pathways. Adapted from [118].

their activity [123], and modulates the redox state of selenocompounds [124]. The thioredoxin system is also implicated in apoptosis, as the oxidation state of thioredoxin regulates apoptosis signaling kinase 1 (ASK1) activity [125]. Through these actions, thioredoxin reductase can regulate redox status and prevent oxidative stress that can contribute to cancer development.

While thioredoxin reductase expression is advantageous in nonmalignant cells for redox balance, it can also benefit cancers. TR1 has been implicated in the malignant and tumor phenotypes of cancer cells, as well as metastasis [126, 127]. TR1 is overexpressed in a number of human tumors, including lung, and is associated with poor prognosis [128-130]. This overexpression has been shown to decrease the sensitivity of cancer cells to electrophilic and oxidative insults, a common mechanism of anti-cancer drugs [131, 132]. TR1 knockdown increases the sensitivity of cancer cells to the anti-cancer drug cisplatin [133, 134] and certain selenocompounds [135]. These studies indicate a role for TR1 in cancer develop, progress, and therapy resistance, making its inhibition a target for cancer therapy.

Glutathione Peroxidases

The glutathione peroxidases are another group of selenoproteins by which selenium can maintain cellular redox state. The glutathione peroxidases are responsible for the majority of glutathione-dependent hydrogen peroxide-reducing activity. There are five selenium-containing glutathione peroxidase isoforms in humans: cytosolic GPx1, gastrointestinal GPx2, plasma GPx3, phospholipid hydroperoxide GPx4, and olfactory GPx6 [95]. Like thioredoxin reductase, expression of these glutathione peroxidases is

selenium dependent. Although GPx2 was originally thought to be expressed only in the gastrointestinal tract, it has since been found to be expressed in other tissues, including the lung. It is the major isoform of glutathione peroxidase induced in the lung in response to cigarette smoke [136], indicating it as the primary glutathione peroxidase involved in lung antioxidant defense.

The Nrf2/ARE Pathway

To date, TR1 and GPx2 are the only two selenoproteins shown to contain an antioxidant response element (ARE). The ARE is a *cis*-acting element located in gene promoters that enhances transcription under conditions of oxidative stress. Activation of ARE gene transcription is regulated by the basic leucine zipper transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2). Several gene classes are Nrf2-regulated, including those with antioxidant, protein folding, cell growth, cell survival, and immune functions [137-141]. In addition to TR1 [142, 143] and GPx2 [136], other antioxidant genes regulated by Nrf2 include NAD(P)H-quinone oxidoreductase 1 (NQO1), [144] the phase II detoxifying glutathione S-transferases [145], UDP glucuronosyltransferases [146], and genes involved in glutathione biosynthesis and reduction [147, 148]. Nrf2 itself has an ARE in its promoter and thus can autoregulate its own expression [149].

The mechanism of Nrf2 activation involves its translocation from the cytoplasm to the nucleus (Figure 1.6). When no ARE inducers are present, Nrf2 remains in the cytoplasm due to an interaction with Kelch-like ECH- associated protein 1 (Keap1) [150]. The Nrf2-Keap1 complex is redox-controlled via thiol groups on Keap1. Keap1 forms a Cul3-Rbx1 E3 ubiquitin ligase complex, allowing for the ubiquitination and

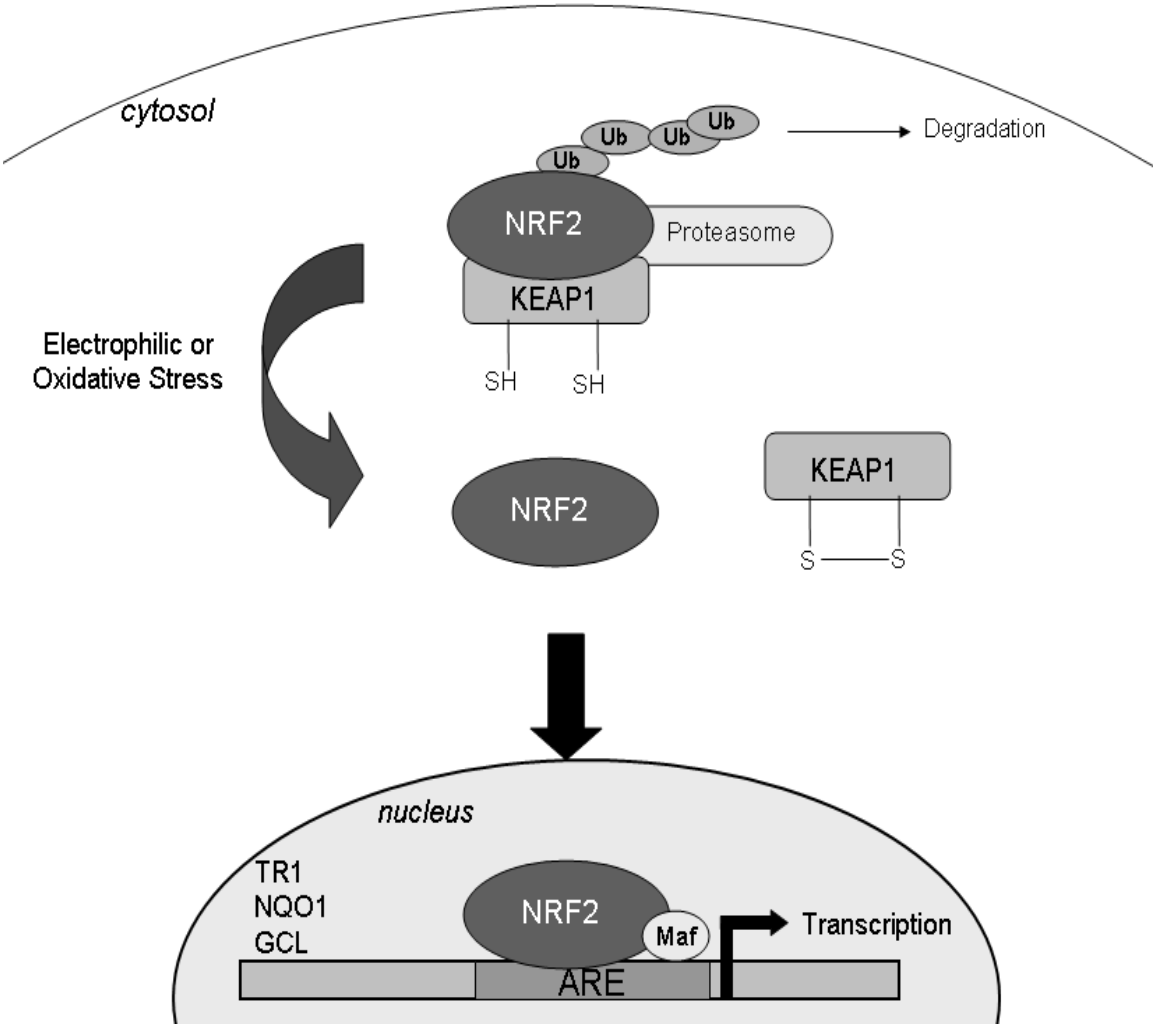


Figure 1.6. Mechanism of Nrf2 activation. Under basal conditions, Nrf2 is tethered to the cytoplasm through an interaction with Keap1, allowing for its ubiquitination and subsequent degradation by the 26s proteasome. Under conditions of electrophilic or oxidative stress, key cysteine residues on Keap1 are modified, releasing Nrf2. Nrf2 can then translocate the nucleus, where it heterodimerizes with small Maf proteins to induce the transcription of ARE genes. Figure adapted from [151].

subsequent degradation of Nrf2 by the 26S proteasome [152]. Under basal conditions, Nrf2 is unstable ($t_{1/2}$ ~ 15 minutes) and only low levels of Nrf2 activation occur [153]. Under conditions of oxidative or electrophilic stress, key cysteine residues on Keap1 are modified. When modification of these cysteine residues occurs, the enzymatic activity of the Keap1-Cul3-Rbx1 E3 complex is inhibited and the Nrf2-Keap1 interaction is disrupted [154]. Dissociation of Nrf2 from Keap1 increases the stability of Nrf2 and allows for it to translocate into the nucleus. Once in the nucleus, Nrf2 heterodimerizes with small Maf proteins [145, 155]. This allows Nrf2 to bind to the ARE, activate the ARE, and increase transcriptional expression of downstream genes [145].

When cellular redox homeostasis is reached, Nrf2 activation is terminated. One model for termination is through a mechanism by which Keap1 translocates to the nucleus. It is thought that Keap1 contains a nuclear export signal that is inactivated under conditions of oxidative stress, but activated when the stress is attenuated [156]. Once in the nucleus, Keap1 dissociates Nrf2 from the ARE and reforms a complex with Nrf2. This Nrf2-Keap1 complex is returned back to the cytosol by a nuclear export sequence in Keap1, where Keap1 again complexes with Cul3-Rbx1 [157] and the degradation of Nrf2 can resume.

Since the discovery of Nrf2-Keap1 complex, other regulatory mechanisms have been associated with Nrf2 activation. These include negative regulation by Bach1 [158], and positive regulation by DJ-1 [159] and KAP1 [160]. The involvement of these other regulators may be specific for the induction of certain downstream genes. Nrf2 phosphorylation has also been implicated, but does not appear to be required for increased Nrf2 stability or nuclear accumulation [161]. While evidence for these other

mechanisms exists, Keap1 modification appears to be the primary means by which Nrf2 activation occurs.

The Nrf2 pathway has been implicated in chemoprevention through its induction of ARE genes. Antioxidant genes can attenuate oxidative stress implicated in carcinogenesis, while phase II metabolizing enzymes can detoxify carcinogens or reactive intermediates. Nrf2 knockout mice are prone to developing more tumors in tissues such as the colon, liver, bladder, skin, and stomach [162]. While the tumorigenicity of lung carcinogens has not been explicitly evaluated in Nrf2 knockout animals, the lungs of Nrf2 knockout mice develop more spontaneous and benzo(a)pyrene-induced genomic DNA mutations [163]. Nrf2 has also been strongly implicated in lung antioxidant defense [164-169], a recognized chemoprevention mechanism. Activation of Nrf2 is one recognized mechanism of chemoprevention by several compounds, including the isothiocyanate sulforaphane [170] and the dithiolethione oltipraz [171]. These findings highlight Nrf2 activation as a therapeutic strategy to enhance antioxidant defense and detoxification pathways, maintain cellular redox homeostasis, and ultimately inhibit carcinogenesis.

Similarly to TR1, Nrf2 can benefit malignancies. Mutations in Nrf2 or Keap1 that lead to constitutively active Nrf2 in the absence of oxidative stress are common in many cancers, including lung [172, 173]. These mutations frequently occur in the regions responsible for binding Nrf2 to Keap1 and prevent the negative regulation of Nrf2 by Keap1. High basal activity of Nrf2 is associated with resistance to anti-cancer drugs [172-175] and its knockdown can increase drug toxicity and basal oxidative stress [174]. When

modulating the Nrf2 pathway for chemoprevention, it is important to take the malignancy status of the tissue into consideration.

Induction of antioxidant genes and phase II enzymes is one hypothesized mechanism of selenium in chemoprevention. The ability of selenocompounds to do so has been demonstrated in both animal tissue and cell culture. Glutathione S-transferase and total GPx activity was increased by one week of dietary *p*-XSC in mouse lung and liver, but no change in UDP-glucuronosyltransferase or selenium-dependent GPx activity was found [175]. *p*-XSC has also been shown to increase glutathione and antioxidant levels in the lung of A/J mice [176]. mRNA expression and activity of antioxidant and phase II enzymes have been assessed in the liver of animals administered selenocystine or the selenazolidines, but no pattern of induction relating to the chemoprevention activity of the compounds was found 24 hours after a single dose [41] or after a week of daily administration [177]. Induction of these enzymes by selenocystine, selenazolidines, and selenomethionine was also observed in Hepa1c1c7 mouse hepatoma cells [178]. Various other selenocompounds have also induced antioxidant and phase II enzymes in tissues [179-183]. It is hypothesized that the generation of reactive species, such as superoxide, by certain selenocompounds may lead to activation of the Nrf2 pathway, but there is currently little data that implicitly demonstrate selenocompound-mediated activation of Nrf2. At present, there is only one published study demonstrating such a mechanism of selenocompounds. This study showed Nrf2 activation by two 3-selena-1-dethiacephem compounds in LNCaP prostate cancer cells [184]. That selenocompounds can induce antioxidant genes and phase II enzymes through the Nrf2 pathway in the lung is one hypothesis to be tested in this work.

Lung Cancer and Selenium

The 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) model of lung cancer has been utilized to evaluate selenocompounds in preventing lung cancer. NNK is a tobacco-specific *N*-nitrosamine carcinogen that forms from nicotine reacting with nitrite. It has a strong affinity for the lung and is the most abundant lung carcinogen in cigarette smoke [185]. NNK must undergo activation via metabolism to exert its carcinogenic effects. The primary metabolism pathway for NNK activation is cytochrome P450-mediated α-hydroxylation of the methylene and methyl carbons adjacent to the *N*-nitroso group [186]. These hydroxylated NNK metabolites are reactive electrophiles that form DNA adducts via methylation or pyridyloxobutylation, leading to tumorigenesis [185, 186]. In addition to being a suspected human lung carcinogen, NNK has been shown to induce lung tumors in rodents independent of the route of administration [185]. These rodent lung tumors have similar pathology as that of human adenocarcinomas [185]. The A/J mouse is one strain sensitive to NNK. In female A/J mice on an AIN-76A diet, a single i.p. dose of 10 μM NNK reproducibly results in 7-12 lung tumors per mouse after 16 weeks [187].

Selenocompounds have been evaluated in the female A/J mouse NNK model with variable success (Table 1.1). *p*-XSC demonstrates activity in this model when present during or after NNK administration [188-190]. When present from pre-initiation to post-initation, selenocystine, OSCA, BSCA, ChSCA, and PhSCA also reduced lung tumor formation [60; 190]. Further studies showed that selenocystine, OSCA, and ChSCA were effective in the post-initiation phase, PhSCA was effective in the pre-initiation phase, and BSCA was effective both pre- and post-initiation but not during NNK administration

Table 1.1. Selenocompound Efficacy at Decreasing Lung Tumor Number in the Female A/J Mouse NNK Model.

Supplementation Period (Relative to NNK Administration)	Dietary Supplementation							
	p-XSC	SECY	ChSCA	OSCA	BSCA	PhSCA	SEM	Se-MSC
Pre, During, and Post	✓[a]	✓[b,c]	✓[c]	✓[b,c]	✓[c]	✓[c]	✗[b]	✗[b]
Pre Only	---	✗[c]	✗[c]	✗[c]	✓[c]	✓[c]	---	---
Post Only	✓[a]	✓[c]	✓[c]	✓[c]	✓[c]	✗[c]	✗[a]	---

'✓' indicates a statistically significant decrease in lung tumor number versus no supplementation.
'✗' indicates no significant decrease in lung tumor number versus no supplementation.
'---' indicates not assessed.

'[a]', Reference 190; '[b]', Reference 60; '[c]', Reference 191.

[191]. Selenized yeast, selenomethionine, Se-methylselenocysteine, selenite, MSCA, PhOSCA and the unsubstituted selenazolidine-4-carboyxlic acid were not effective when given from the pre-initiation phase to the post-initiation phase [60, 188-191]. MSA has yet to be evaluated in an animal model of lung cancer, but has elicited changes in gene expression of lung cancer cells *in vitro* that could be beneficial in lung cancer prevention [92]. These studies support the use of selenium in lung cancer prevention and demonstrate that its effects are compound-dependent.

Gene profile analyses of lung cells or tissue have been performed to delineate what mechanisms may be associated with the selenocompounds. Microarray analysis of H460 lung cancer cells treated with *p*-XSC showed changes in transcription factor, growth factor, and apoptotic gene expression [192]. MSA altered the expression of regulatory cell cycle proteins involved in the G_1 to S phase transition in H520 lung cancer cells, resulting in G_1 phase arrest [92]. This profile of MSA in lung differed slightly from that observed in prostate cancer cells, with fewer cell cycle proteins modulated in the NSCLC cells [92]. Lung tissue from mice provided selenocystine or the selenazolidines OSCA, BSCA and ChSCA in the diet for 10 days following NNK administration was also assessed by microarray analysis. With these treatments, only six genes were found to be differentially expressed compared to animals receiving no selenium supplementation [191]. However, these genes demonstrated modest changes and were not good indicators of selenium supplementation. No changes in the transcription factor, apoptotic or cell cycle genes altered by *p*-XSC or MSA were observed with selenocystine and certain selenazolidines, indicating a differential mechanism of these compounds. Additionally, selenocompounds may affect different gene profiles in the malignant cells used for the

p-XSC and MSA studies compared to nonmalignant tissue utilized for the selenocystine and selenazolidine study.

Clinical selenium supplementation trials in humans have had mixed results in regard to lung cancer. The initial follow-up of the NPC trial showed a significant benefit of selenium in decreasing lung cancer incidence and mortality (Relative Risk = 0.54, $p = 0.04$) [63]. A trend towards decreasing risk was found with extended follow-up for the whole population, but was not statistically significant (Relative Risk = 0.70, $p = 0.18$) [70]. A significant decrease in incidence remained for the low tertile baseline plasma selenium group (Hazard Ratio = 0.42, $p = 0.04$). No difference in risk was found between former and current smokers supplemented with selenium. SELECT found no effect of selenomethionine in the total population, but has not had the data for low baseline selenium population analyzed for lung cancer incidence. However, selenomethionine has not demonstrated efficacy in animal models and perhaps other selenocompounds that are more efficacious in these models may demonstrate a benefit in human trials. Other studies evaluating serum or toenail selenium status and lung cancer risk have not conclusively shown a protective effect of high selenium levels [193], but a meta-analysis of thirteen of these epidemiological studies has indicated populations with low selenium levels as the population which benefits most from selenium [193]. Again, these findings emphasize the selenocompound form and baseline selenium levels as important factors in determining the benefits of selenium in lung cancer prevention.

A Phase III clinical trial is currently underway to investigate the ability of selenium as selenized yeast to prevent new tumor growth in patients with previously resected Stage I non-small cell lung cancer (ClinicalTrials.gov Identifier NCT00008385).

This trial began enrolling patients in October 2000 and is estimated to be completed in November 2014. It will be interesting to see if participants have a similar benefit with selenium to that which was seen for vitamin A and whether any observed benefits correlate with baseline selenium status.

Taken together, these studies indicate a role for selenium in lung cancer, but the mechanisms are not well understood. The ability of selenium to serve in the antioxidant defense system may be particularly advantageous in lung cancer prevention. Oxidative stress, especially from tobacco smoke, has been strongly linked to the pathogenesis of lung cancer and attenuation of this stress by selenium could be a beneficial mechanism. Additionally, targeting selenium-dependent redox regulation in lung cancer, such as through TR1 inhibition, may be useful in anti-cancer therapy to promote an oxidized redox state and cell death. Further studies into mechanisms of selenium, such as the ones detailed in this work, are necessary to provide greater understanding as to how cancers can be most effectively prevented and treated.

Research Objectives

The overall objective of this research is to determine the redox mechanisms of selenium in malignant and nonmalignant human lung cancer cells. The hypothesis for this work is that distinct selenocompounds alter the redox status in the lung, with Nrf2/ARE pathway activation and TR1 playing a role in this modulation. The effects of various selenocompounds on cellular redox status in the lung are currently unknown and may relate to the cellular metabolism of a given compound and malignancy status. It is thought that selenium may contribute to chemoprevention in nonmalignant lung cells by

priming the antioxidant defense system with low levels of redox stress, and in effect prepare the antioxidant defense system to attenuate oxidative stress and detoxify potential carcinogens. In malignant cells, it is anticipated that TR1 is able to mitigate the redox effects of selenocompounds and decreasing its expression will shift cellular redox status to the oxidative side and enhance selenocompound-induced cell death. As several selenocompounds are assessed in each objective, this work also seeks to provide evidence for which form of selenium might be most efficacious in the lung. The findings of this work will be applicable to the design of future cancer prevention and treatment studies.

Major Findings

Chapter 2

Parameters of cellular redox status were assessed in malignant A549 human adenocarcinoma cells and nonmalignant BEAS-2B human bronchial epithelial cells. The results indicate that selenium has disparate effects that are both selenocompound and cell line-specific. Selenocystine and ChSCA elicited a moderate oxidative stress at 24 hours in A549 cells, but not BEAS-2B cells. MSA produced a reductive stress. Selenomethionine had no effect on the parameters evaluated in either cell line. The selenocompounds investigated also demonstrated greater toxicity in the BEAS-2B line at this time point. Additional findings from this work indicate that the basal redox status of malignant A549 human adenocarcinoma cells tends to be more reduced in comparison to nonmalignant BEAS-2B human lung cells. The difference in basal redox state of these two cell lines may explain the differential effects of the selenocompounds in each line.

Chapter 3

The ability of selenocompounds to induce antioxidant and glutathione-related genes in the lung was assessed. Selenocystine showed increased expression of genes related to glutathione metabolism (glutathione S-transferases, GPx2) and oxidoreductase activity (TR1, NQO1) in the lung tissue of mice provided selenocystine in the diet for seven days after NNK administration. Expression of TR1, NQO1, and the glutathione biosynthesis gene glutamate cystine ligase catalytic subunit (GCLc) was increased with selenocystine, ChSCA, MSA, and selenomethionine in BEAS-2B cells. These compounds initially produced reactive oxygen species and depleted glutathione, but restored intracellular glutathione to levels at or above baseline by 24 hours. Induction of TR1, NQO1, and GCL_c by selenocystine, ChSCA, and MSA was shown to be Nrf2 regulated, as these compounds increased nuclear Nrf2 protein expression and the induction of gene expression was attenuated in Nrf2 knockdown cells. No increase in nuclear Nrf2 protein expression was found at the same time point for selenomethionine. This work concludes that selenocompounds may initially function as prooxidants to induce ARE genes through an Nrf2 mechanism, ultimately increasing cellular antioxidant capacity.

Chapter 4

In A549 human adenocarcinoma cells, TR1 knockdown enhanced the toxicity of selenocystine, BSCA, and ChSCA at 48 hours, but not MSA, selenomethionine or the anticancer drug cisplatin. TR1 knockdown also increased the ability of selenocystine and the selenazolidines to generate reactive oxygen species. Enhanced cytotoxicity with these

compounds was associated with their ability to deplete intracellular total glutathione to levels less than 20% of baseline. The cell death mechanism was caspase-independent and shown to involve mitochondrial dysfunction, DNA strand breaks, and release of apoptosis inducing factor from the mitochondria. The findings from this work emphasize inhibition of both the TR and glutathione antioxidant systems as a novel treatment strategy for lung cancer.

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CHAPTER 2

MODULATION OF REDOX STATUS IN HUMAN LUNG CELL LINES BY ORGANOSELENOCOMPOUNDS

Abstract

Cancer prevention strategies utilizing selenium-containing compounds have demonstrated reduced cancer mortality and efficacy for some cancer types but the types of selenocompounds evaluated have been limited. The literature suggests that there are considerable differences in cellular effects among selenocompounds observed in preclinical studies. We used human lung cell lines, A549 and BEAS-2B, to evaluate the effect on cell viability, redox modulation, and disruption of subcellular compartments by the conventional selenium-containing amino acid, selenomethionine, and compare its effects with other organoselenocompounds, including selenocysteine prodrugs, which have demonstrated provocative anticancer activity in preclinical lung tumor models. In these studies, we observed little effect on the cells with selenomethionine treatment, evidence of reductive stress with methylseleninic acid, and mild oxidative stress with certain selenocysteine prodrugs in the adenocarcinoma cell line but the effects were attenuated in the normal, but virally transformed cell line. These data support the notion that the form of the organic selenocompound is a major determinant in the expected

cellular response to the selenocompound and that perhaps other novel selenocompounds warrant additional study as they may demonstrate improved performance compared to selenomethionine.

Introduction

Selenium is a micronutrient that is required for the synthesis of selenoenzymes, including some that are essential for embryogenesis [1-3]. Selenium plays a role in human health, with deficiencies in the diet predisposing individuals to certain rare diseases such as Keshan disease [4] as well as increase susceptibility to cancer [5]. However, selenium has also been considered toxic and can even be carcinogenic in certain forms [6]. The mechanisms for selenium effects in cancer are not well understood and most likely vary with the selenocompound. Nevertheless, several studies have demonstrated that selenium supplementation attenuates cancer mortality and disease [7, 8] and historically, in the United States, dietary selenium levels in forage crops have inversely correlated with cancer mortality [9, 10].

While the relationship between selenium and cancer is complex, the Nutritional Prevention of Cancer (NPC) trial is the seminal study to date that identified a selenium cancer prevention benefit by demonstrating an overall decrease in cancer mortality with particular benefits to colon, lung, and prostate cancer incidence [11]. Follow up evaluation of NPC trial participants maintained a selenium benefit in prostate and a marginal benefit for colon cancer but the lung cancer benefit has not remained statistically significant [12, 13]. The NPC trial utilized selenium in the form of selenized yeast, where most of the selenium is in the form of the selenoamino acid

selenomethionine [14]. The results of the NPC trial were instrumental for the largest ongoing cancer prevention trial, the Selenium and Vitamin E Cancer Prevention Trial (SELECT) [15, 16].

The biochemistry of selenium and selenocompounds is of interest due to their redox properties. However, the cancer prevention activities and toxicities of these compounds vary considerably in lung tumor model systems. The inorganic selenium compound, sodium selenite, has shown minimal activity while organic selenocompounds like 1,4-phenylenebis(methylene)selenocyanate (*p*-XSC), and certain selenium containing amino acids, including selenocystine and many selenazolidines (selenocysteine prodrugs) have demonstrated anticancer activity [17-21]. It is of interest that the form of selenium most frequently utilized in cancer prevention trials, selenomethionine, as well as a methylated form of selenocysteine, Se-methylselenocysteine, also show minimal activity in lung cancer model systems [17, 18]. In human lung cell lines various selenocompounds have demonstrated anticancer activities; for example, *p*-XSC and methylseleninic acid (MSA) induced cell cycle arrest and apoptosis [22, 23]. Therefore, it does not appear that it is merely selenium itself, but perhaps instead, the metabolism and biological utility of the selenocompound that will dictate which selenocompounds will be most useful for cancer prevention [24].

Since the mechanisms for selenium-mediated cancer prevention appear to vary among selenocompounds and as there is an unexplained variability in chemopreventive efficacy that does not correlate with selenium levels alone, more studies are needed to evaluate the activity of distinct selenocompounds to determine which compounds may provide the most benefit without unwanted toxicity. In this study, we contrast the effects

of several classes of selenocompounds, including *p*-XSC, methylseleninic acid, selenoamino acids and selenazolidines on cellular viability, thiol status, generation of reactive oxygen species, and mechanisms of toxicity in the human lung cell lines A549 and BEAS-2B.

Materials and Methods

Materials

Materials used included L-selenomethionine and L-selenocystine from Acros Organics (Morris Plains, NJ). 2-oxoselenazolidine-4(R)-carboxylic acid (OSCA) and 2-cyclohexylselenazolidine-4-(R)-carboxylic acid (ChSCA) were synthesized as described [25, 26] (supported by USPHS Grant No. GM058913). *p*-XSC was from LKT Laboratories, Inc (St. Paul, MN) and MSA was from PharmaSe, Inc. (Lubbock, TX). Cell Counting Kit-8 was from Dojindo Molecular Technologies (Gaithersburg, Maryland). Monoclonal antibodies directed against BiP/GRP78 and α-tubulin were purchased from Becton Dickinson and Company (Franklin Lakes, NJ) and Zymed Laboratories, Inc. (South San Francisco, CA), respectively, and donkey polyclonal anti-mouse antibodies conjugated with horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bovine serum albumin standard and Coomassie Plus Protein Reagent were from Pierce Biotechnology (Rockford, IL). Human fibronectin and bovine collagen were purchased from Fisher Scientific (Houston, TX). LHC-8 medium, Advanced (DMEM), monobromobimane (mBBr), 2,7-dichlorofluroescein diacetate (DCFH-DA), propidium iodide (PI), 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1), NuPAGE 10% Bis-Tris gels and

bovine serum albumin were purchased from Invitrogen (Carlsbad, CA). Protease inhibitor cocktail tablets (complete) were purchased from Roche (Indianapolis, IN). PVDF membrane was purchased from Millipore (Burlington, MA). Western Lighting chemiluminescence reagents were from PerkinElmer Life Sciences (Boston, MA). Dimethylsulfoxide (DMSO), retinoic acid, and epinephrine were purchased from Sigma-Aldrich (St. Louis, MO). Common buffers and salts were purchased from Sigma-Aldrich, Fisher Scientific or VWR scientific.

Cell Lines and Culture Conditions

The A549 cell line was purchased from the American Type Culture Collection and was cultured in Advanced Dulbecco's modified essential medium supplemented with 2% fetal bovine serum and glutamine. BEAS-2B cells (gift from Dr. Christopher Reilly, University of Utah) were cultured in LHC-8 medium supplemented with 0.33 nM retinoic acid and 2.75 μM epinephrine. To facilitate adhesion, a plate coat of LHC medium supplemented with 1 mg/100 ml human fibronectin, 1 mg/100ml bovine collagen, and 0.75 g/100ml bovine serum albumin was applied to BEAS-2B cell culture flasks and plates prior to seeding cells. Both cell lines were maintained at 37^{o}C in an atmosphere of 5% CO_2 and 95% air, with passages made once cells reached ~ 80-90% confluence.

Selenocystine and selenomethionine were dissolved in the appropriate cell culture medium for the cell type. All other selenocompounds were dissolved in DMSO. Final concentrations of DMSO for selenocompounds treatments where DMSO served as vehicle were less than or equal to 0.1%.

Viability Assay

Cellular viability was determined using a Cell Counting Kit-8 (CCK-8). Briefly, cells were seeded into 48-well plates at 2-4 × 10^4 cells/well and allowed to recover overnight. Cells were then treated with selenocompounds in log increment concentrations for 24 hours. Following the treatment period, medium was aspirated and replaced with 4% CCK-8 in the appropriate cell culture medium. Absorbance at 460 nm and 650 nm was measured after incubation at 37^{o}C until the reagent developed sufficiently for maximal reading using a Perkin-Elmer VictorV3 Multimode Microplate Reader. Sample absorbance, measured at 650 nm was subtracted from the 460 nm absorbance to ensure that the measurements were not affected by sample turbidity.

Cytometric Assays for Redox Metrics

Cells were seeded into 6-well plates at a density of ~ 2 × 10^5 cells/well and allowed to grow overnight. Culture medium was refreshed at the time of treatment. Cellular fluorescence concentrations were determined 24 hours after treatment using a Beckman Cell Lab Quanta SC flow cytometer by dividing the fluorescence for each cell by its measured electronic volume. For each assay, a minimum of 10,000 events per sample was recorded.

Free Thiols: Cells were trypsinized, centrifuged at 250×*g* for 5 minutes, and resuspended in 1 ml phosphate buffered saline (PBS). Cells were then centrifuged at 250×*g* for 5 minutes and resuspended in a fresh 1 ml of 1× PBS. 40 μM monobromobimane (mBBr) was added and samples were incubated at room temperature in the dark for 5 minutes. mBBr fluorescence concentration was then determined.

Reactive Oxygen Species: 10 μM 2,7-dichlorofluroescein diacetate (DCFH-DA) was added to media of cells in 6-well plates following selenocompound treatment and incubated at 37^{o}C with 5% CO_2 and 95% air for 30 minutes. Cells were trypsinized, centrifuged at 250×*g* for 5 minutes, and resuspended in 1 ml PBS. 2 μg/ml propidium iodide (PI) was then added to the cell suspension to distinguish between viable and compromised cell populations. Both DCF and PI fluorescence were measured for each sample. DCF fluorescence concentration from PI negative (viable) cells was used for reported results.

Subcellular Organelle Targets

Cells were seeded into 6-well plates at a density of ~ 2 × 10^5 cells/well and allowed to grow overnight. Culture medium was refreshed at the time of treatment. Selenocompound effects were measured by evaluation of the mitochondrial potential and immunochemical hybridization to determine expression levels of the ER chaperonin BiP/GRP78 as a marker of the unfolded protein response.

Mitochondrial potential: 1 μM JC-1 was added to medium of cells attached to 6-well plates after selenocompound treatment. Cells were incubated at 37^{o}C with 5% CO_2 and 95% air for 20 minutes and then trypsinized, washed and resuspended as described above for cytometric analysis. JC-1 fluorescence at 525 nm (JC-1 “green”) and 575 nm (JC-1 “red” or “J-aggregates”) was determined for each sample. JC-1 fluoresces green when the mitochondrial potential has been depolarized and forms aggregates that fluoresce red when the mitochondrion is polarized. Carbonyl cyanide

3-chlorophenylhydrazone (CCCP) was used at 25 μM to disrupt the mitochondrial membrane potential as a positive control.

BiP/GRP78 Western blot: Cells in 6-well plates were placed on ice. Media was aspirated and cells were then washed with 1ml of cold 1× PBS and the PBS aspirated. 100 μl of a lyses buffer containing 50 μl of 25× complete protease inhibitor cocktail, 12.5 μl of 10% sodium dodecyl sulfate, 1187.5 μl of a buffer containing 50 mM Tris pH 7.4, 100 mM NaCl, and 2 mM EDTA was added to each well and cells scraped. Lysate was transferred to 1.5 ml microfuge tubes and sonicated 10× using a 40% duty cycle on an ultrasonic processor. Lysates were centrifuged 10,000×g for 10 minutes at 4^{o}C and the supernatants transferred. Protein concentrations were determined using Bradford reagents. Absorbance at 595 nm was measured using a Perkin-Elmer VictorV3 Multimode Microplate Reader and sample concentrations were determined using a bovine serum albumin standard curve. The membrane was probed with primary anti-BiP/GRP78 antibody at a 1:1000 dilution overnight at 4°C. Membranes were then washed with washing buffer (5 mM Tris pH 8.0, 150 mM NaCl, 2.7 mM KCl, 0.1% Tween-20), probed with secondary donkey anti-mouse IgG horseradish peroxidase antibody at a 1:5000 dilution for 45 minutes at room temperature, and washed again. For detection of α-tubulin, membranes were incubated with stripping buffer (62.5 mM Tris pH 6.7, 2% sodium dodecyl sulfate, 0.7% β-mercaptoethanol) at 50^{o}C for 1 hour and washed. Membranes were probed with anti-α-tubulin at a 1:500 dilution overnight at 4^{o}C, washed, and probed with secondary donkey anti-mouse IgG horseradish peroxidase antibody as described above. Protein was detected using Western Lightning Western Blot Chemiluminescence reagent and visualized on a Kodak Image Station 440.

Statistical Analysis

1-way ANOVA was used to determine statistical significance between control and treatment values (GraphPad InStat Version 3.06). Dunnett's multiple comparisons post hoc testing was used to establish significance among the distinct selenocompound treatment groups compared with the control samples with $p < 0.05$ considered significant.

Results

Toxicity of Selenocompounds in Lung Cells

Differential toxicity of the selenocompounds was observed between the BEAS-2B and A549 cell lines (Figure 2.1). The A549 cells demonstrated greater resistance to toxicity for the majority of the selenocompounds, exceptions being *p*-XSC and higher doses of selenocystine. In the more sensitive BEAS-2B cells, *p*-XSC was highly toxic (100% lethal at > 10 μM), followed by selenocystine. The selenazolidines, ChSCA and OSCA demonstrated only minor toxicity at doses >100 μM. In the BEAS-2B cells, the selenazolidines showed an enhancement of cell viability compared with control at doses <20 μM. Within the 24 hour time period of evaluation, MSA-treated cells displayed decreased viability at low doses (<2 μM) in both cell lines, but the viability decrease did not follow the expected sigmoidal dose response curve. Selenomethionine showed very little (BEAS-2B) or no (A549) toxicity.

Alterations in Cellular Redox Parameters

Since selenium is redox active, the cellular redox state was determined following exposure to selenocompounds using cytometric assays. To avoid confusion due to high

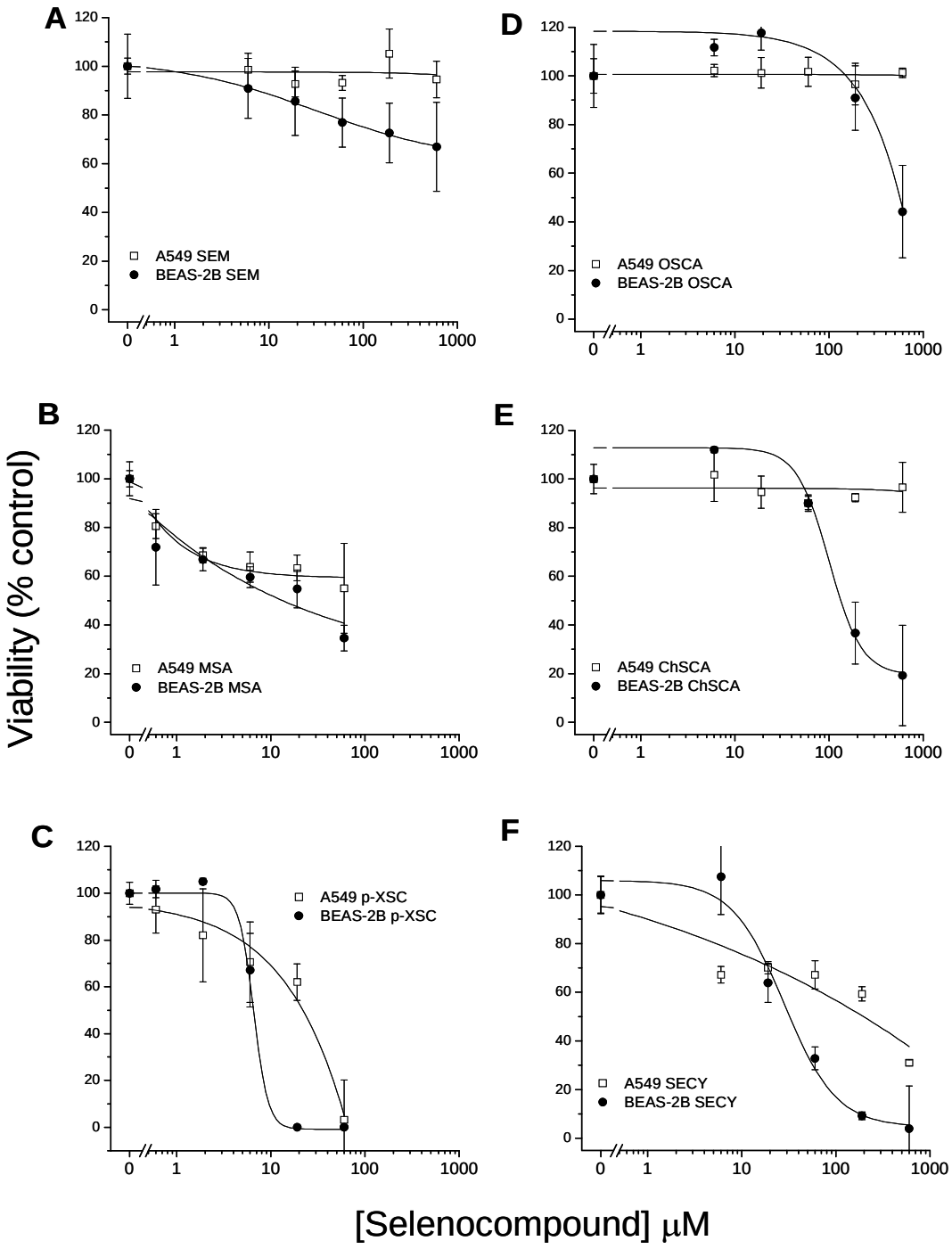

Figure 2.1. Human lung cell line viability following treatment with selenocompounds. Dose response curves for A549 cells (open squares) and BEAS-2B cells (filled circles) treated with: **(A)** SEM, **(B)** MSA, **(C)** *p*-XSC, **(D)** OSCA, **(E)** ChSCA, and **(F)** SECY for 24 hours. *p*-XSC and MSA were evaluated at concentrations between 0 to 60 μM while all other selenocompounds were evaluated at concentrations between 0 to 600 μM. Symbols represent the mean viability of triplicate measures as a percentage of control with standard deviations.

cell death, selenocompound concentrations that resulted in less than 25% loss of viability were selected for use in further experiments. Since *p*-XSC was considerably more toxic than the other compounds assessed, it was not included in these or any further analyses.

Cellular free thiol levels were determined as a measure of reducing capacity in a time-dependent assay using the fluorophore monobromobimane (mBBr, Figure 2.2). As thiol levels can change with cell size, the fluorescence concentration was normalized to the electronic volume of the cells. After a 24 hour incubation with 2.5 μM MSA, both cell lines showed significant increases in mBBr fluorescence. Selenomethionine treatment resulted in an increase in mBBr fluorescence in the A549 cells, but not in the BEAS-2B cells. In contrast to MSA, ChSCA and selenocystine exposure produced a significant decrease in mBBr fluorescence. The other selenazolidine investigated, OSCA, had no statistically significant effect in either cell line.

Cellular reactive oxygen species (ROS) levels were measured by the oxidation of DCFH to the fluorophore DCF (Figure 2.3). Again, the fluorescence concentration of cells treated with the selenocompounds was normalized to electronic cell volume. Only 100 μM selenocystine resulted in a significant alteration in cellular ROS within this 24 hour period and only in A549 cells. However, we observed a considerable difference in the basal DCF fluorescence between these cell lines suggesting that BEAS-2B cells have higher basal ROS levels.

It should be noted that these alterations in cellular redox status do not appear to result from a direct effect of the compounds since cellular redox status changes were not seen when cells were evaluated after 4 hours of exposure (data not shown). The changes develop over time and similar results were found at 16 and 24 hours (the 24 hour data are

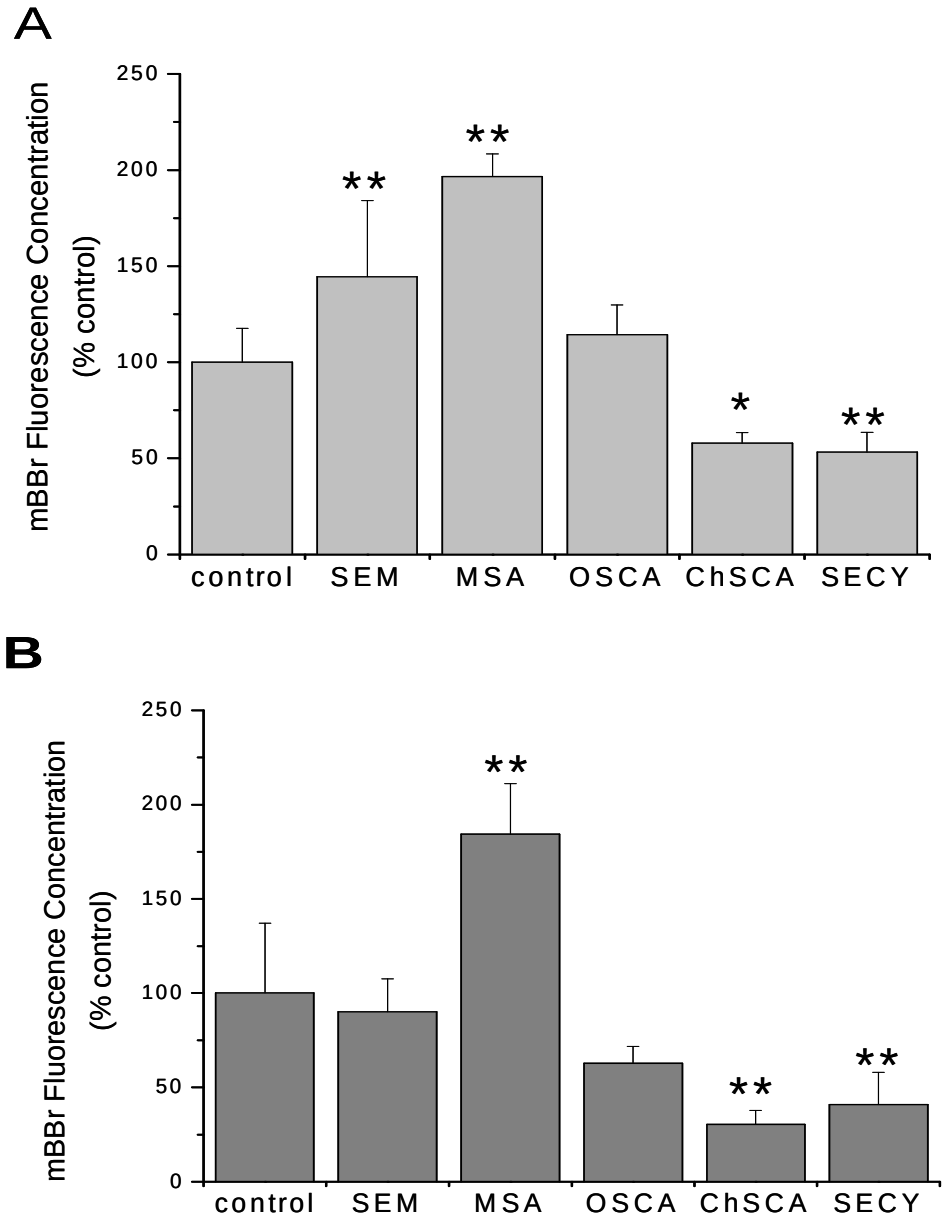

Figure 2.2. Thiol status following treatment with selenocompounds in human lung cells. Analysis of **(A)** A549 cells and **(B)** BEAS-2B cells using the fluorophore mBBr as a measure of cellular thiol status. Cells were incubated with 40 μM mBBr for 5 minutes prior to cytometric analysis. Selenocompounds were used at concentrations that resulted in less than 25% decreases in viability. Concentrations used were: SEM, 100 μM; OSCA, 100 μM; MSA, 5 μM (A549) or 2.5 μM (BEAS-2B); ChSCA, 100 μM (A549) or 50 μM (BEAS-2B); SECY, 100 μM (A549) or 25 μM (BEAS-2B). Bars represent the mean mBBr fluorescence concentrations normalized to the control with standard deviations. The control mean mBBr fluorescence concentrations were 0.267 and 0.203 with coefficient of variation of 13% and 12% for A549 and BEAS-2B, respectively. Samples with significant differences from the control are marked with asterisks: *, $P<0.05$; **, $P<0.01$.

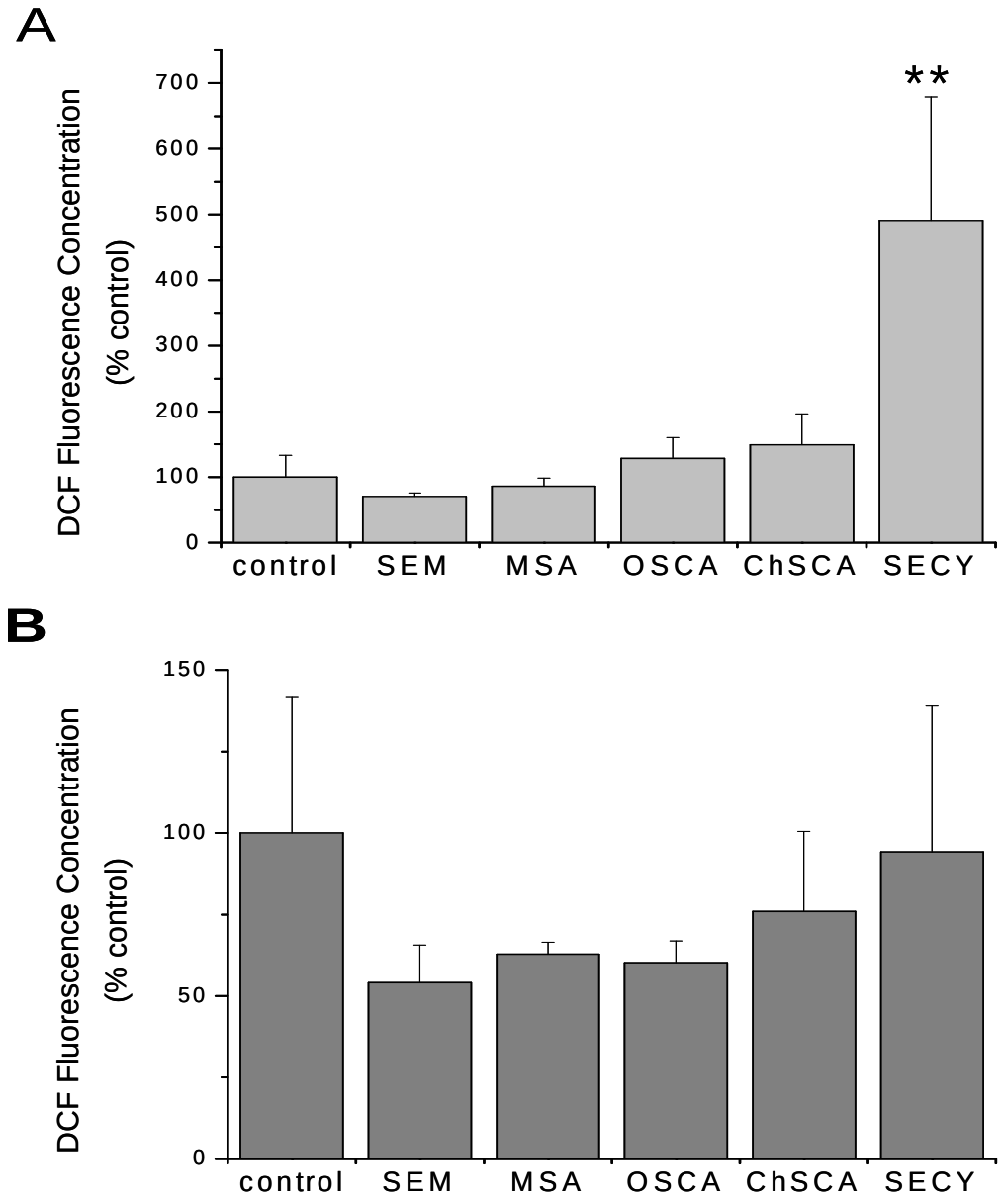

Figure 2.3. Generation of ROS in human lung cell lines following treatment with selenocompounds. Analysis of **(A)** A549 cells and **(B)** BEAS-2B cells using the oxidation sensitive fluorophore DCFH as a measure of cellular ROS. Cells were incubated with DCFH-DA for 30 minutes and then incubated with PI prior to cytometric analysis so that only PI-negative cells were assessed for DCF fluorescence. Cells were exposed to the selenocompounds for 24 hours. Concentrations used were: SEM, 100 μM; OSCA, 100 μM; MSA, 5 μM (A549) or 2.5 μM (BEAS-2B); ChSCA, 100 μM (A549) or 50 μM (BEAS-2B); SECY, 100 μM (A549) or 25 μM (BEAS-2B). Bars represent the mean DCF fluorescence concentrations normalized to the control with standard deviations. The control mean DCF fluorescence concentrations were 0.009 and 1.398 for A549 and BEAS-2B, respectively. Only SECY in the A549 cells demonstrated a significant difference (**, $P<0.01$) compared to the control.

shown in Figures 2.2 and 2.3).

Effects of Selenocompounds on Subcellular Organelles

To better understand the mechanisms of toxicity seen in Figure 2.1, selenocompound effects on the mitochondria and endoplasmic reticulum (ER) were evaluated. Of the selenazolidines, only ChSCA was used in these experiments since OSCA-treated cells displayed minimal toxicity (Figure 2.1) and did not show significant differences in thiol status (Figure 2.2) or ROS (Figure 2.3).

The mitochondrial potential was measured cytometrically using JC-1 (Figure 2.4). In these experiments, a high degree of consistency between cell lines was observed. Selenomethionine and MSA treatments did not alter the mitochondrial potential in either cell line. Selenocystine and ChSCA treatments both depolarized the mitochondrial membrane to an extent similar to the mitochondrial potential disrupter CCCP.

ER stress has been identified as a consequence of exposure to certain selenocompounds [27, 28]. For these studies, BiP/GRP78 protein expression was used as a marker of ER stress. BEAS-2B cells appear to have higher basal expression of BiP/GRP78 than A549 cells and there was less modulation of the protein level in BEAS-2B cells. This higher level of Bip/GRP78 protein expression in BEAS-2B cells is consistent with the higher basal ROS levels observed above (Figure 2.3). In A549 cells, MSA, selenocystine, and ChSCA induced BiP/GRP78 protein expression (Figure 2.5). In BEAS-2B cells, minor changes in BiP/GRP78 expression were observed but not consistently among multiple experiments. Selenomethionine did not appear to induce ER stress in either cell line.

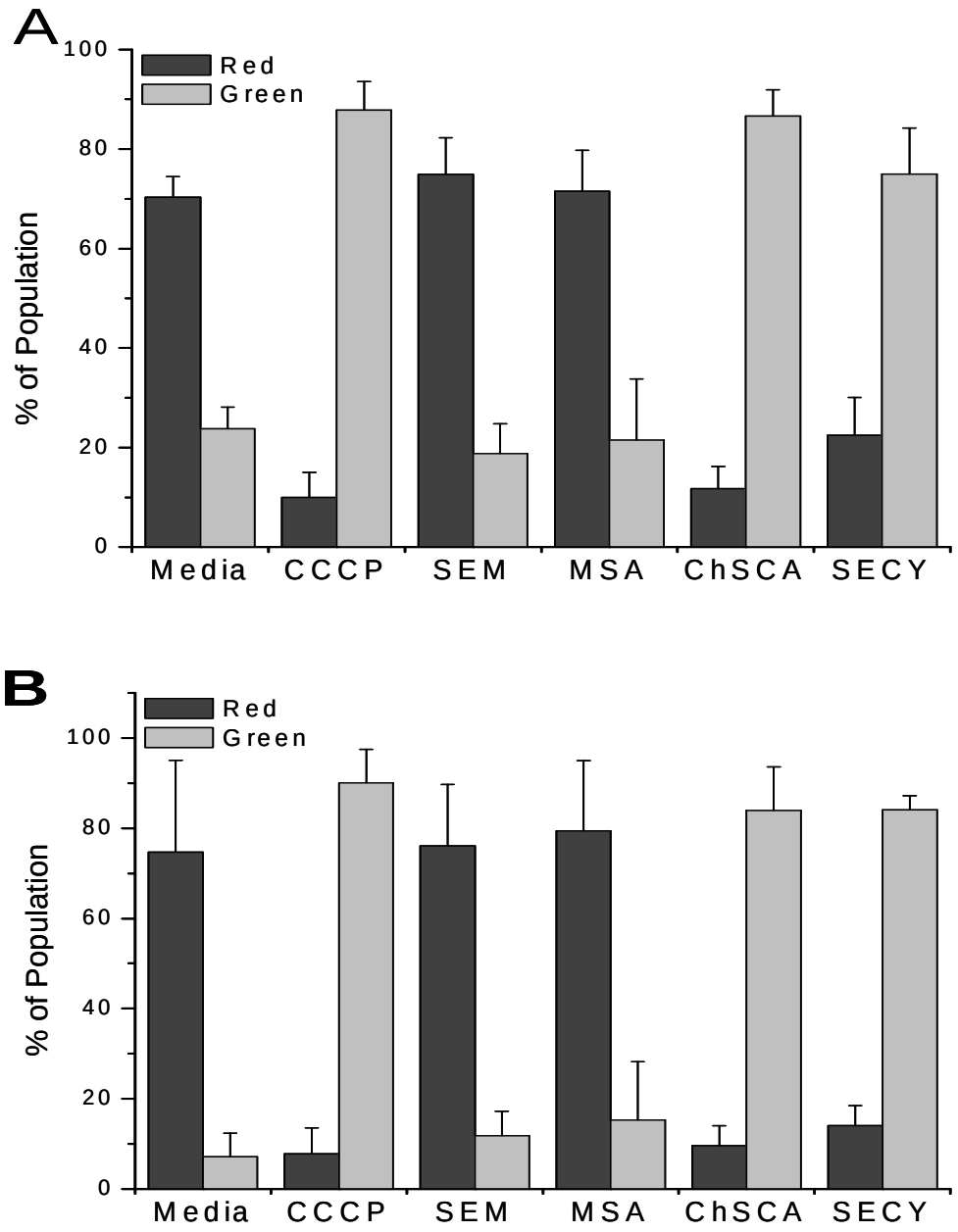

Figure 2.4. Depolarization of mitochondrial membrane potential by selenocompounds. Analysis of **(A)** A549 cells and **(B)** BEAS-2B cells using the mitochondrial fluorophore JC-1 to cytometrically measure the mitochondrial membrane potential. CCCP (25 μM), a recognized mitochondrial membrane potential disrupter, was utilized as a positive control. Cells were incubated with selenocompounds for 24 hours. The concentrations of the compounds used were: SEM, 100 μM; MSA, 5 μM; ChSCA, 200 μM (A549) or 100 μM (BEAS-2B); SECY, 100 μM (A549) or 50 μM (BEAS-2B). Bars represent the percentage of the cellular population with polarized mitochondria as indicated by red fluorescence (525 nm) or depolarized mitochondria as indicated by green fluorescence (575 nm) with standard deviations. Treatment with CCCP, ChSCA, and SECY resulted in a significant difference from control, with $P<0.001$.

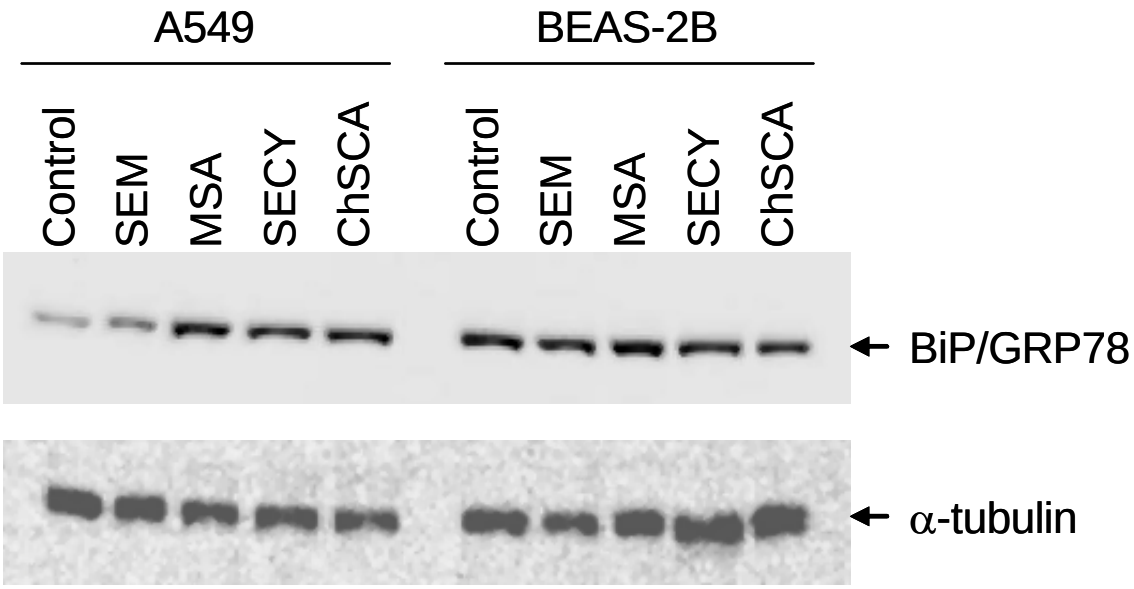

Figure 2.5. Induction of BiP/GRP78 expression by selenocompounds in human lung cells. As an indicator of ER stress and the unfolded protein response, BiP/GRP78 was evaluated by immunoblot (a representative blot of replicate experiments is shown). Cell lysates were homogenized and 10 μg of protein was loaded onto a NuPAGE 10% Bis-Tris gel and transferred to a PVDF membrane for immunochemical analysis as described in the Methods. Cells were incubated with the selenocompounds for 24 hours. The concentrations of the compounds used were: SEM, 100 μM; MSA, 5 μM; ChSCA, 200 μM (A549) or 100 μM (BEAS-2B); SECY, 100 μM (A549) or 50 μM (BEAS-2B). Immunochemical analysis of α-tubulin was utilized as a loading control.

Discussion

Selenium compounds can display differential effects in cancer and noncancer cells; however the mechanisms that delineate these differences are not clear. Previous studies have demonstrated selenium-mediated effects in premalignant and neoplastic rat and canine mammary cells but minimal effects in normal cells [29, 30]. In addition, another study has demonstrated differences between normal and transformed human cell lines treated with selenite [31]. In the experiments described herein, A549 cells, a commonly used lung adenocarcinoma-derived cell line, and BEAS-2B cells, normal lung epithelial cells virally transformed for immortal growth in culture that generally display a non-tumorigenic phenotype [32, 33], were utilized to examine selenocompound selective sensitivity. We observed differences in the viability and redox modulation by distinct selenocompounds between these cells, and these differences may reflect the basal redox states that reflect the mutational status of KEAP1, a negative regulator of NRF2, an activator of antioxidant genes, in these cells [34].

Distinct selenocompounds have also demonstrated unexplained variability in cancer prevention model systems. In human lung cell lines, various selenocompounds have demonstrated anticancer activities; for example, *p*-XSC and MSA induced cell cycle arrest and apoptosis [22, 23], while selenomethionine enhanced the radiation-mediated cell killing [35]. These studies are consistent with potential antitumor activities. Nevertheless, in the widely used murine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) lung tumor model, inorganic sodium selenite was ineffective [17, 18] while the organic selenocompound, *p*-XSC, reduced the tumor burden in NNK-induced lung cancer in mice and rats [17, 19, 20]. Certain selenium containing amino acids, including

selenocystine and many selenazolidines (selenocysteine prodrugs), were effective against NNK-induced lung tumors [18, 21]. However, a methylated form of selenocysteine, Se-methylselenocysteine, lacked activity in this model [17, 18]. Selenomethionine, the currently preferred agent in human selenium cancer prevention studies, does not show efficacy at chemoprevention in studies in animal models of lung cancer [18, 36] and has not proven effective as a cancer prevention agent in the follow-up studies of human lung cancer [12, 13]. In contrast to MSA and the selenazolidines, selenomethionine did not modulate redox parameters measured in the experiments herein, except a modest increase in cellular thiol levels in A549 cells (Figures 2.2 and 2.3), and did not affect the mitochondrion or ER subcellular compartments. A possible explanation for the lack of efficacy of selenomethionine may be due to misincorporation in protein synthesis [12], i.e., replacing methionine, rather than metabolic conversion of the selenium component into either newly synthesized selenoproteins or methylselenol. Other selenocompounds that have demonstrated preclinical efficacy in lung cancer models await clinical scrutiny.

The mechanisms of MSA-mediated cancer prevention have been rigorously pursued and indicate that the methylselenol metabolite is crucial for activity [37]. Mechanistic studies point to induction of the unfolded protein response (UPR) in the ER as a likely cellular locus of action [27, 28]. Our data with MSA are consistent with these previous studies and support the hypothesis that it generates a reductive stress, which was observed by increased monobromobimane fluorescence in both A549 and BEAS-2B cells (Figure 2.2). This stress on the ER was validated by the increase in BiP/GRP78 protein expression, particularly in A549 cells (Figure 2.5).

Of the selenium-containing amino acids, the diselenide selenocystine was clearly the most toxic and demonstrated an oxidative activity in cells by decreasing free thiols in both A549 and BEAS-2B cells (Figure 2.2), as well as overtly increasing ROS levels in A549 cells (Figure 2.3). The selenocysteine prodrugs, OSCA and ChSCA, were considerably less toxic than selenocystine (Figure 2.1), and their redox modulation of the lung cell lines may reflect the differences in the expected metabolism of these compounds. ChSCA is expected to spontaneously hydrolyze to selenocysteine while OSCA is expected to require enzymatic degradation by 5-oxo-L-prolinase to release selenocysteine [26]; OSCA displayed less redox modulation than ChSCA, especially in A549 cells. Indeed, there are reports of oxoprolinase levels differing in the tissue of normal lung and lung cancer, with oxoprolinase expression decreasing in lung cancer compared to normal lung tissue [38]. The cellular redox alterations observed with ChSCA may suggest that a more rapid release of selenocysteine from ChSCA may generate some selenocystine.

Based on the differences in redox modulation by distinct selenocompounds, we suggest that these differences may provide a basis for distinct mechanisms of action in tumor models. Of the redox modulating selenocompounds, we speculate that the selenocystine and the selenazolidines may activate an antioxidant response while MSA activates a reductive stress pathway. The selenazolidines show particular promise due to their minimal toxicity and cancer prevention activity.

Acknowledgments

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CHAPTER 3

SELENOCOMPOUND INDUCTION OF ANTIOXIDANT GENES IN THE LUNG VIA THE NRF2/ARE PATHWAY

Abstract

Organoselenium compounds have been shown to decrease lung tumor incidence in tobacco carcinogen animal models, such as the NNK model. Selenium supplementation trials and epidemiological studies have also indicated a potential benefit of selenium in lung cancer, although the chemoprevention mechanism of selenium in the lung is not well understood. Here we show that independent of NNK administration, selenocystine induces mRNA expression of genes associated with glutathione metabolism and oxidoreductase activity in the lungs of female A/J mice. To further investigate the mechanism of gene induction, we utilized the BEAS-2B human lung cell line and found that selenocystine, the selenocysteine prodrug ChSCA, MSA, and the common nutritional supplement, selenomethionine all increased reactive oxygen species levels and depleted intracellular glutathione at 2 hours. Selenocystine, ChSCA, and MSA, but not selenomethionine, increased nuclear Nrf2 protein expression at 4 hours. Nevertheless, all the selenocompounds increased both TR1 mRNA and protein expression, and NQO1 mRNA at 24 hours. With the exception of selenomethionine, the compounds increased intracellular glutathione levels at 24 hours. ChSCA and MSA also

increased expression of these genes in normal human bronchial epithelial cells, while none of the compounds modulated expression in A549 adenocarcinoma cells. Nrf2 knockdown inhibited the ability of the selenocompounds to induce antioxidant gene mRNA in BEAS-2Bs. Taken together, these findings indicate that although the time course and cell line sensitivity may differ among compounds, organoselenium compounds can increase cellular antioxidant capacity through the Nrf2/ARE pathway and for those with chemopreventive activity in lung, establishes Nrf2 activation as a potential mechanism.

Introduction

Selenium in the form of several organic compounds has been investigated as an agent for lung cancer prevention. Selenocystine and certain selenazolidines (selenocysteine prodrugs) have demonstrated efficacy in reducing the number of lung tumors formed in the NNK mouse model of lung tumorigenesis [1, 2]. *p*-XSC has also demonstrated efficacy in the NNK model, while sodium selenite and, notably, selenomethionine were ineffective [1, 3]. Se-methyl-L-selenocysteine, the methylated cysteine congener of selenomethionine, was also ineffective [1]. Methylseleninic acid (MSA), a methylselenol precursor, has yet to be evaluated in an animal model of lung cancer, but has exhibited antiproliferative activity in lung cell culture [4]. These studies show that selenium can function as a chemopreventive agent in the lung, but efficacy is compound dependent. The NPC trial has also indicated a potential benefit for selenium in decreasing lung incidence and mortality [5]. The mechanism of chemoprevention and which form of selenium might be most efficacious both remain under investigation.

It is well established that cigarette smoke contains reactive oxygen and nitrogen species that can place a large oxidative burden on the lung. Oxidative stress contributes to the initiation and progression of lung carcinomas [6] and a chemoprevention strategy to combat oxidative insult is to increase the antioxidant defense system of the lung. Activation of the Nrf2 pathway is one mechanism by which antioxidant capacity can be enhanced. Nrf2 is basic leucine zipper transcription factor that under basal conditions is tethered to the cytoplasm by its negative regulator Keap1, where it remains inactive and is targeted for proteasomal degradation by ubiquitation [7, 8]. Under conditions of oxidative or electrophilic stress, Nrf2 dissociates from Keap1 and can then translocate to the nucleus. Once in the nucleus, Nrf2 associates with small Maf proteins and binds to ARE sequences located in gene promoters to initiate transcription. A battery of antioxidant and phase II detoxifying genes are recognized as being regulated by Nrf2 [9], including the antioxidant enzymes NQO1 [10], TR1 [11, 12], and GPx2 [13]; the glutathione-S-transferase (GST) and UDP glucuronosyltransferase (UGT) detoxification enzymes [9, 14, 15]; and the glutamate cysteine ligase catalytic (GCLc) and modulatory subunits required for glutathione biosynthesis [16]. Thus, Nrf2 activation serves as a method by which the inherent cellular antioxidant defense can be enhanced to attenuate oxidative stress, such as that implicated in lung cancer development.

Previous work focused on the lung has been shown that certain selenocompounds have the ability to modulate redox parameters *in vitro* [17] and decrease tumor burden *in vivo* [1, 2]. These alterations in cellular redox status coupled with the observed chemopreventitive activity led us to investigate whether Nrf2 is involved in the response of the lung to organoselenium compounds. We demonstrate herein that selenocystine,

ChSCA and MSA increase endogenous antioxidant and glutathione biosynthesis gene expression in the lung in an Nrf2-dependent manner.

Materials and Methods

Reagents

L-selenocystine and DMSO were purchased from Sigma–Aldrich (St. Louis, MO), L-selenomethionine was from Acros Organics (Morris Plains, NJ), MSA was purchased from PharmaSe, Inc. (Lubbock, TX), R-sulforaphane [1-isothiocyanate-(4R)-(methylsulfinyl)butane] was from LKT Laboratories (St. Paul, MN), and ChSCA was synthesized as previously described [18]. AIN-76A diet was from Dyets, Inc. (Bethlehem, PA) and NNK was obtained from Toronto Research (Toronto, Canada). BEAS-2B cells were a gift from Dr. Christopher Reilly (University of Utah). Advanced DMEM, LHC-8 media, LHC basal media, First Strand cDNA synthesis kit with SuperScriptIII, H_2DCFDA, PI, NuPAGE Bis-Tris gels, Lipofectamine 2000, and Opti-MEM I reduced-serum media were from Invitrogen (Carlsbad, CA). SYBR Green I Master Mix and Complete protease inhibitor tablets were from Roche (Indianapolis, IN). Bovine serum albumin standard, Coomassie Plus Protein Reagent, SuperSignal chemiluminescence reagent, and tris(2-carboxyethyl)phosphine hydrochloride (TCEP HCl) were from Thermo Scientific (Rockford, IL). Primary monoclonal antibody against TR1 and primary polyclonal antibodies against Nrf2 (C-20), lamin B1 and GAPDH were from Santa Cruz Biotechnology (Santa Cruz, CA). NQO1 polyclonal primary antibody was purchased from Abcam (Cambridge, MA). Secondary horseradish peroxidase (HRP)-conjugated antibodies were from Santa Cruz Biotechnology or CalBioChem (San

Diego, CA). GSH-Glo and CellTiter-Fluor kits were purchased from Promega (Woods Hollow, WI). Primers for QPCR and siRNA oligos were synthesized at the University of Utah DNA/Peptide synthesis core facility. All other reagents were purchased from established suppliers.

Cell Culture

BEAS-2B cells were cultured in LHC-8 media supplemented with 2.75 μM epinephrine and 0.33 nM retinoic acid. The normal human bronchial epithelial (NHBE) primary cell line with retinoic acid was purchased from Lonza and cultured in bronchial epithelial growth media (BEGM). A549 cells were from ATCC and were cultured in advanced DMEM supplemented with 1% Glutamax and 2% fetal bovine serum. Prior to seeding, BEAS-2B and NHBE cell culture surfaces were pretreated with a plate coat consisting of LHC basal media, 10 μg/ml bovine serum albumin, 10 μg/ml fibronectin, and 30 μg/ml collagen. NHBE cells at or below passage number 6 were utilized for experiments. For cell treatments the following concentrations were used: 100 μM selenomethionine, 2.5 μM MSA, 1 μM (BEAS-2B), 5 μM (NHBE), or 100 μM (A549) selenocystine, 5 μM (NHBE), 25 μM (BEAS-2B) or 50 μM (A549) ChSCA, and 1 μM (NHBE), 5 μM (BEAS-2B) or 10 μM (A549) SF. Treatments were performed at concentrations where cells demonstrated ≥75% viability after 24 hours [17; 19-21]. DMSO severed as vehicle for all treatments at a concentration ≤ 0.1%.

Animals and Lung Tissue RNA Collection

Female A/J mice at 5 weeks of age were obtained from the Jackson Laboratory (Bar Harbor, ME). Animals were housed on hardwood bedding in a temperature and humidity controlled environment on a 12 hour light/dark cycle and given free access to food and water. Animals were stabilized on an unsupplemented AIN-76 diet for 1 week prior to being given the selenium supplemented diet. The basal level of selenium in the diet is 0.35 ppm selenium. NNK was administered i.p. as a single 10 μM injection in 0.2 mL saline. With this protocol, 100% of animals reproducibly develop lung tumors after 3 months [22]. Three days after NNK administration, animals were provided AIN-76A diets supplemented with selenocystine at 15 ppm selenium ad libitum for 10 days. This 15 ppm concentration in the diet has demonstrated little toxicity while decreasing lung tumor incidence when given during the pre- and post-initiation periods and the post-initiation period alone in the NNK model [1, 2]. Fresh preparations of the selenocystine diet mix were provided to animals daily. Animal procedures were approved by the University of Utah Animal Care and Use Committee and conducted in agreement with NIH laboratory animal care guidelines.

Animals were sacrificed thirteen days after NNK administration. Lung tissue was harvested, immediately homogenized in Trizol and frozen. RNA was purified by the Trizol protocol followed by Qiagen RNeasy clean-up and then quantified on a Nanodrop ND-1000 spectrophotometer. The quality of the RNA samples collected was determined using the Experion Automated Electrophoresis Station (Biorad) with the RNA StdSens Analysis Kit. All samples displayed RNA Quality Index levels greater than 9.

Microarray Expression Analysis

Microarray gene expression analysis was performed using Agilent 44K Whole Mouse Genome Oligonucleotide Microarrays in a manner similar to how we have previously analyzed microarray data [23]. Agilent labeling kits were used to generate Cy-dye labeled cRNA. The four no-treatment samples were combined and used as a standard reference to phenotypically anchor the dataset. Slides were hybridized and then scanned with the Agilent Microarray Scanner equipped with Feature Extraction software (FEv8.1). This software preprocesses the data as follows: local background is subtracted, irregular spots flagged, and global linear regression (lowess) normalization is performed, and this ratio is log transformed. We imported the data into TIGR MEV 3.1 software for further analysis. A supervised strategy was used to identify the genes with the greatest significant differences among untreated, NNK, selenocystine and NNK plus selenocystine treatment groups using multiclass Significance Analysis of Microarrays (SAM). Five-hundred iterations were used in the SAM analysis to evaluate the false discovery rate and a conservative cutoff was used (median false discovery rate of 0%) to identify differentially expressed genes. Gene Ontology analysis of the differentially expressed genes was assessed with Expression Analysis Systematic Explorer (EASE). The gene expression profiles for the genes highlighted by the EASE assessment were hierarchically clustered for visualization.

Quantitative Real Time PCR

Total RNA was isolated from cells using the Qiagen RNAeasy kit. First stand cDNA synthesis was performed using 1 μg total RNA and 150 ng/μL random primers

with SuperScriptIII according to the manufacturer's protocol. QPCR was performed using a Roche LightCycler 480 for 40 cycles at 95^{o}C for 10 s, 65^{o}C for 5 s, and 72^{o}C for 10 s. Each amplification reaction contained a 1:80 dilution of cDNA, 500 nM primer, and SYBR Green I Master Mix. Target gene mRNA expression was normalized to beta-2 microglobulin (β2M) mRNA expression. Melt curve analysis was performed after amplification to ensure the generation of a single product. The following primer sets were utilized:

TR1 forward: 5'- TTGGAATCCACCCTGTCTGT-3'

TR1 reverse: 5'-CATCCACACTGGCTTAAC-3'

NQO1 forward: 5'-ATGTATGACAAAGGACCCTTCC-3'

NQO1 reverse: 5'-TCCCTTGCAGAGAGTACATGG-3'

GCL_C forward: 5'-ATGCCATGGGATTTGGAAT-3',

GCL_C reverse: 5'-AGATATACTGCAGGCTTGGAATG-3'

β2M forward: 5'-TTCTGGCCTGGAGGCTATC-3'

β2M reverse: 5'-TCAGGAAATTTGACTTTCCATTC-3'.

Reactive Oxygen Species

Reactive oxygen species were assessed following 2 hour treatments by incubating cells with 20 μM H_2DCFDA for 20 minutes at 37^{o}C. 2',7'-dichlorofluorescin (DCF) and PI fluorescence concentrations were determined cytometrically as previously described, with a minimum of 10,000 events collected for each sample [17].

Total Intracellular Glutathione

Glutathione concentrations were determined after 2 hour treatments using the GSH-Glo kit as previously described [24]. Reduced glutathione (GSH) was directly measured. Total glutathione (GSSG + GSH) was measured by adding 1 mM TCEP. GSH concentrations were normalized to the live cell fluorescence values obtained using the CellTiter-Fluor reagent.

Immunoblot Analysis

Whole cell lysates were collected in passive lyses buffer as previously described [17]. Nuclear lysates were obtained with the Active Motif Nuclear Extract Kit following the manufacturer's protocol. Protein concentrations were determined using the Bradford method. Equal amounts of protein were run out on 10% or 4-12% NuPAGE Bis-Tris gels and transferred to PVDF membranes. Membranes were blocked with 5% milk in T-BST and probed with the following primary antibody dilutions: 1:200 anti-Nrf2, 1:200 anti-lamin B1, 1:300 anti-TR1, 1:800 anti-NQO1, 1:500 anti-GAPDH. When probing for Nrf2, membranes were incubated with primary antibody overnight, fixed in 0.25% glutaraldehyde in cold PBS for 15 minutes at 4^{o}C, and blocked again in 5% milk. Membranes were washed with T-BST, probed with HRP conjugated secondary antibodies, and washed again with T-BST. Protein was detected using chemiluminescence and blots were visualized on a Kodak ImageStation.

Nrf2 Knockdown by siRNA

BEAS-2B cells were seeded at 5.5 x 10^4 cells/well and allowed to recover overnight. Cells were transfected with 200 nM Nrf2 siRNA [25] or control siRNA that does not code for any known human gene (5'-AGGCAAAUCACGGUGUCCUtt-3', sense sequence) using Lipofectamine 2000 in OptiMEM serum free medium. After 24 hours, the transfection mixture was removed and LHC-9 media containing the treatment compounds was added. Treatments were performed for 24 hours, after which whole cell lysates for immunoblotting and total RNA for QPCR were collected as described above.

Statistical Analysis

Data are presented as mean ± standard deviation. Two-way ANOVA with Holm-Sidak post-hoc testing was used to analyze the mRNA expression data for BEAS-2B Nrf2 knockdown and control cells. All other data were evaluated using one-way ANOVA Holm-Sidak post-hoc testing. $p < 0.05$ was considered significant. SigmaStat Version 3.5 was used for analysis.

Results

Induction of Antioxidant and Phase II Genes in Mouse Lung

Gene expression in mouse lung RNA was assessed 13 days after NNK administration in untreated, NNK-only, selenocystine-only, and selenocystine + NNK treated animals. We chose this early time point after carcinogen administration but before tumorigenic changes occur to gain insight into effects selenocystine may have on the lung

in this premalignant stage. No tumors or pathology were observed when the lungs were harvested.

Of the >41,000 data elements on the murine whole genome microarrays, only 60 elements representing 54 unique genes were identified by multiclass SAM as differentially expressed among the four treatment groups designated as untreated, NNK, selenocystine, and NNK plus selenocystine. No changes in genes related to NNK metabolism, such as cytochrome P450s, were observed at this time point following selenocystine consumption. Of the genes induced, enrichment of those related to GSH metabolism and oxidoreductase activity was indicated by EASE analysis in the selenocystine and selenocystine + NNK animals (Figure 3.1). Little induction of these genes occurred in the untreated and NNK-only animals. This indicates that increased expression of this set of genes is in response to the selenocystine and is unrelated to NNK. Several Gst classes were induced, including the alpha, mu, and pi isoforms. These enzymes catalyze the formation of GSH conjugates with a variety of xenobiotics. Gpx2, an enzyme that utilizes GSH to reduce hydroperoxides, was also induced. Thioredoxin reductase 1 (Txnrd1) is one of the primary regulators of cellular redox status, contributing to overall oxidative tone and redox-dependent signaling. Selenocystine exposure also increased expression of the detoxifying enzymes Nqo1, Ugt1a6b, carboxylesterase 1 (Ces1), sulfiredoxin 1 (Srxn1) and aldehyde dehydrogenase family 1 member A1 (Aldh1a1), indicating an increased capacity of the lung to metabolize oxidative and reactive species. All these genes induced by selenocystine are known to be regulated by the Nrf2 transcription factor.

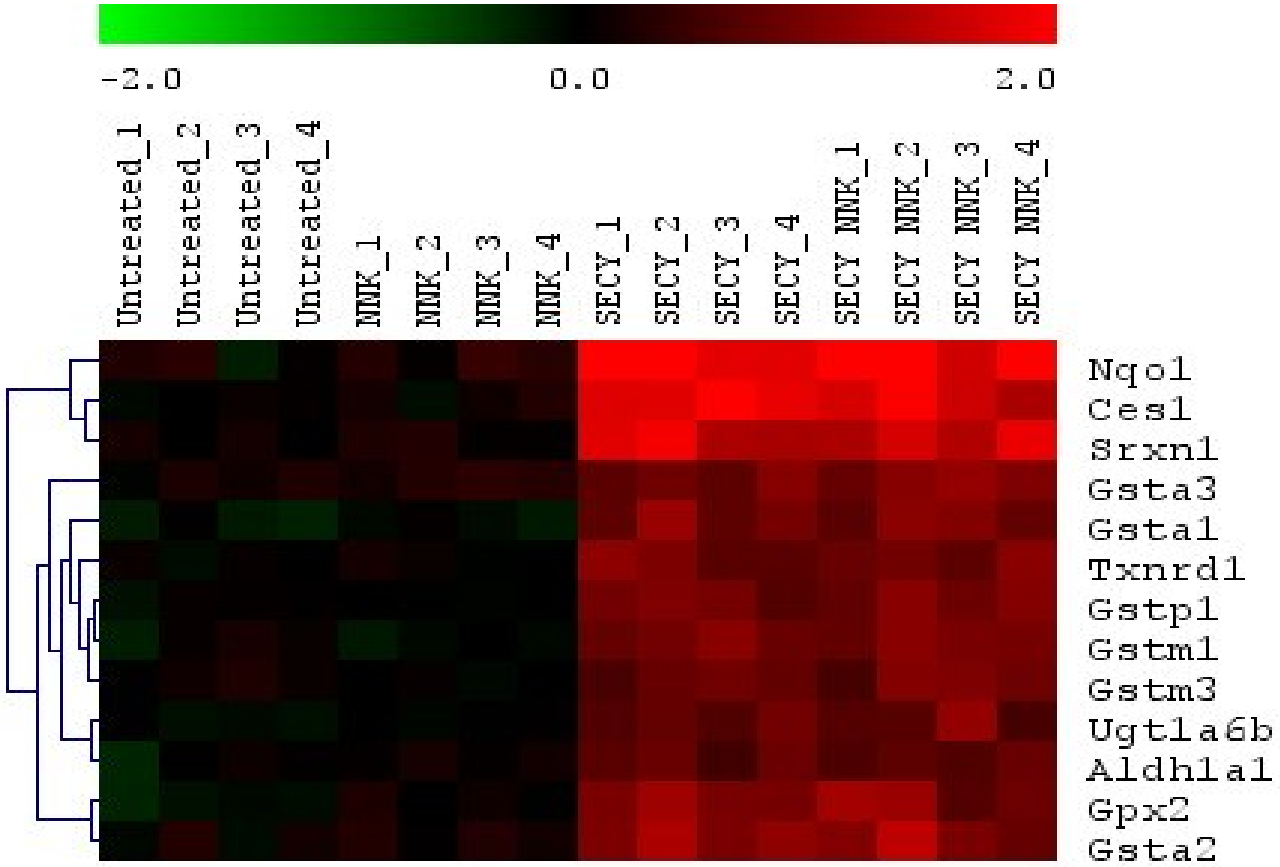

Figure 3.1. Heatmap of mRNA expression in mouse lung by selenocystine. Mice were injected with NNK and 3 days later provided diets supplemented with selenocystine (SECY) at 15 ppm selenium for 10 days. Lung tissue was then harvested and RNA purified for whole genome microarray gene expression analysis. Using multiclass SAM, only 60 elements distinguished the gene expression among four groups: untreated, NNK-treated, SECY, and SECY plus NNK. EASE analysis of those genes identified glutathione metabolism and oxidoreductase activity as significantly enriched. The heatmap displays the relative gene expression among four biological replicates. The scale bar at the top represents transcription levels (in $\log_2$) from 4-fold down to 4-fold increased gene expression.

Induction of Nrf2 Target Genes in Human Lung Cells

To investigate the involvement of Nrf2 in the mechanism of antioxidant gene induction that was observed in the mouse lung, we utilized the human lung BEAS-2B cell line. BEAS-2Bs are a human nonmalignant bronchial epithelial cell line transformed with adenovirus. We were interested in whether induction of this class of genes is specific to selenocystine, or also occurs with the selenocysteine prodrug ChSCA, the selenoamino acid selenomethionine, or the hypothesized methylselenol precursor MSA. The isothiocyanate sulforaphane (SF), a known Nrf2 activator and phase II enzyme inducer, served as the positive control for cell culture experiments [26].

All selenocompounds increased the mRNA expression of two antioxidant genes, the selenoenzyme TR1 and the nonselenium containing antioxidant gene NQO1, at 24 hours, with ChSCA increasing mRNA levels to the greatest extent (Figure 3.2A, B). Increases in TR1 protein expression were also observed with all treatments. However, protein levels did not quantitatively correlate with the level of mRNA induction (Figure 3.2C, D). ChSCA increased TR1 mRNA expression to the greatest extent, but selenocystine produced the greatest increase in TR1 protein expression. Modest increases in NQO1 expression were observed with all treatments. These data suggest that the induction of TR1 and NQO1 is quite dynamic for mRNA but more modest for protein. Overall, the organoselenium compound induction of protein for both TR1 and NQO1 was similar in magnitude to the positive control, sulforaphane.

The ability of the selenocompounds to induce glutathione biosynthesis was also assessed. Messenger RNA expression of GCL_C, the catalytic subunit for the rate limiting step of glutathione synthesis [27] was significantly increased by selenocystine, ChSCA,

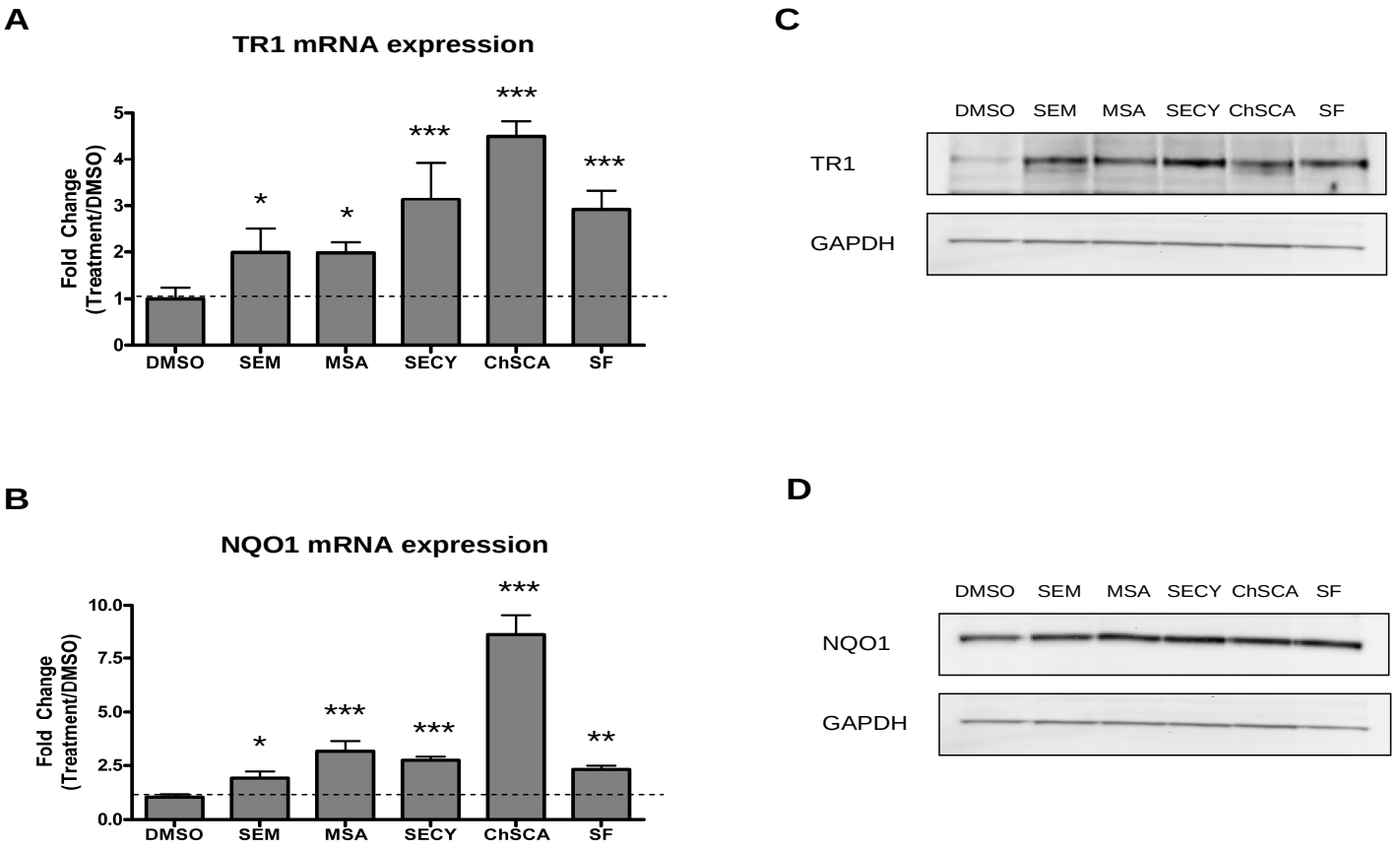

Figure 3.2. Induction of Nrf2 regulated antioxidant genes in BEAS-2B cells following 24 hour treatment. (A) TR1 and **(B)** NQO1 mRNA expression. Data are presented as mean fold change relative to DMSO control. n=3 for all treatments. Treatments were 100 μM selenomethionine (SEM), 2.5 μM methylseleninic acid (MSA), 1 μM selenocystine (SECY), 25 μM 2-cyclohexylselenazolidine-4-carboxylic acid (ChSCA) and, the positive control, 5 μM sulforaphane (SF). Significant differences are denoted by: '*', '**', and '***" indicate $p<0.05$, $p<0.01$, and $p<0.001$ versus DMSO control, respectively. **(C)** TR1 and **(D)** NQO1 protein expression as determined by Western blotting. GAPDH was probed as protein loading control. Immunoblots shown are representative of at least two experimental replicates.

selenomethionine, and the positive control, sulforaphane (Figure 3.3A). The 2-fold increase in GCL_C mRNA elicited by MSA treatment was not statistically significant at the p=0.05 level. The intracellular glutathione content was also assessed to determine the possible functional consequence of GCL_C mRNA induction. We measured both intracellular GSH (reduced glutathione) and GSH + GSSG (total glutathione) levels to gain insight into the relative redox status of glutathione as a measure of overall oxidative tone. Selenocystine, ChSCA and MSA significantly increased glutathione levels to a greater extent than sulforaphane (Figure 3.3B). Similar increases occurred for both total and reduced GSH, that is, the GSH/GSSG ratio did not markedly change. Selenomethionine was unique in that the increased GCL_C mRNA expression did not result in increased GSH or GSSG content above baseline levels. MSA was able to elicit an increase in GSH and GSSG content despite only the minor two fold increase in GCL_C mRNA expression.

Antioxidant gene mRNA expression was also assessed in NHBE cells, another model of the nonmalignant lung, to determine broad applicability of selenocompound effects. The changes in gene expression occurred earlier in the NHBE cells for all selenocompounds compared to the BEAS-2B cells so effects were primarily monitored after 12 hours. While selenocystine did not change mRNA expression of TR1, NQO1, and GCL_C in this cell line, the selenocysteine prodrug, ChSCA produced a robust response in all three genes (Figure 3.4A). Selenomethionine did not induce any of these genes in NHBE cells, while MSA increased expression of all three although the 4-fold increase in GCL_C did not achieve statistical significance. The positive control,

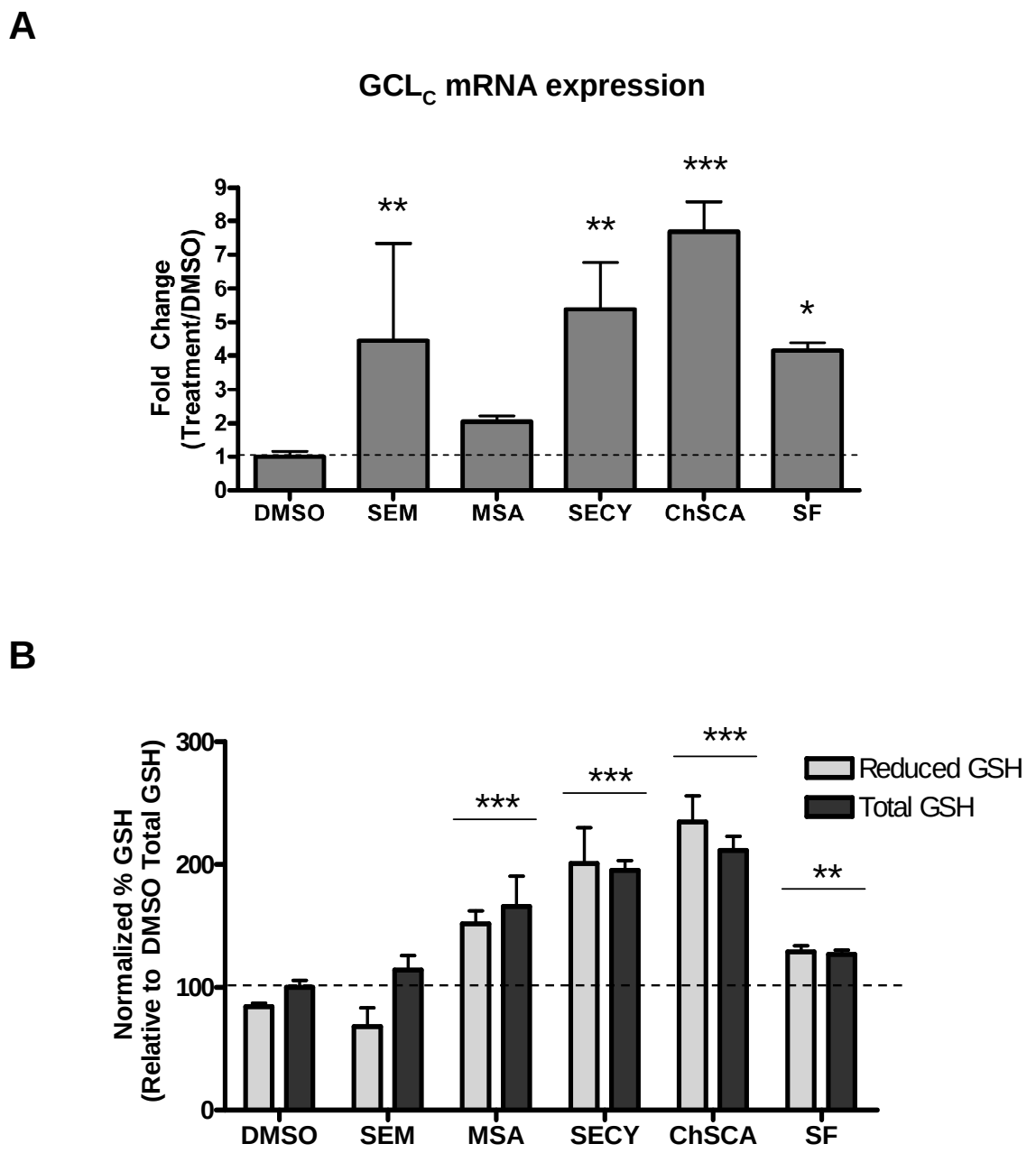

Figure 3.3. Glutathione is increased in BEAS-2B cells after 24 hour selenocompound treatment. Treatment concentrations were 100 μM SEM, 2.5 μM MSA, 1 μM SECY, 25 μM ChSCA, and 5 μM SF. **(A)** GCL_C mRNA expression. Data are presented as mean fold change relative to DMSO control. n=3 for all treatments. Selenocompound treatments are as denoted in Figure 3.2. Significant differences are denoted by: '*', '**', and '***" indicate $p<0.05$, $p<0.01$, and $p<0.001$ versus DMSO control, respectively. **(B)** Reduced and total intracellular glutathione levels. Data are normalized to cell viability fluorescence measurements and are presented as mean % total glutathione DMSO control. n=4 for all treatments. '**' indicates $p<0.01$ and '***' indicates $p<0.001$ versus respective DMSO control.

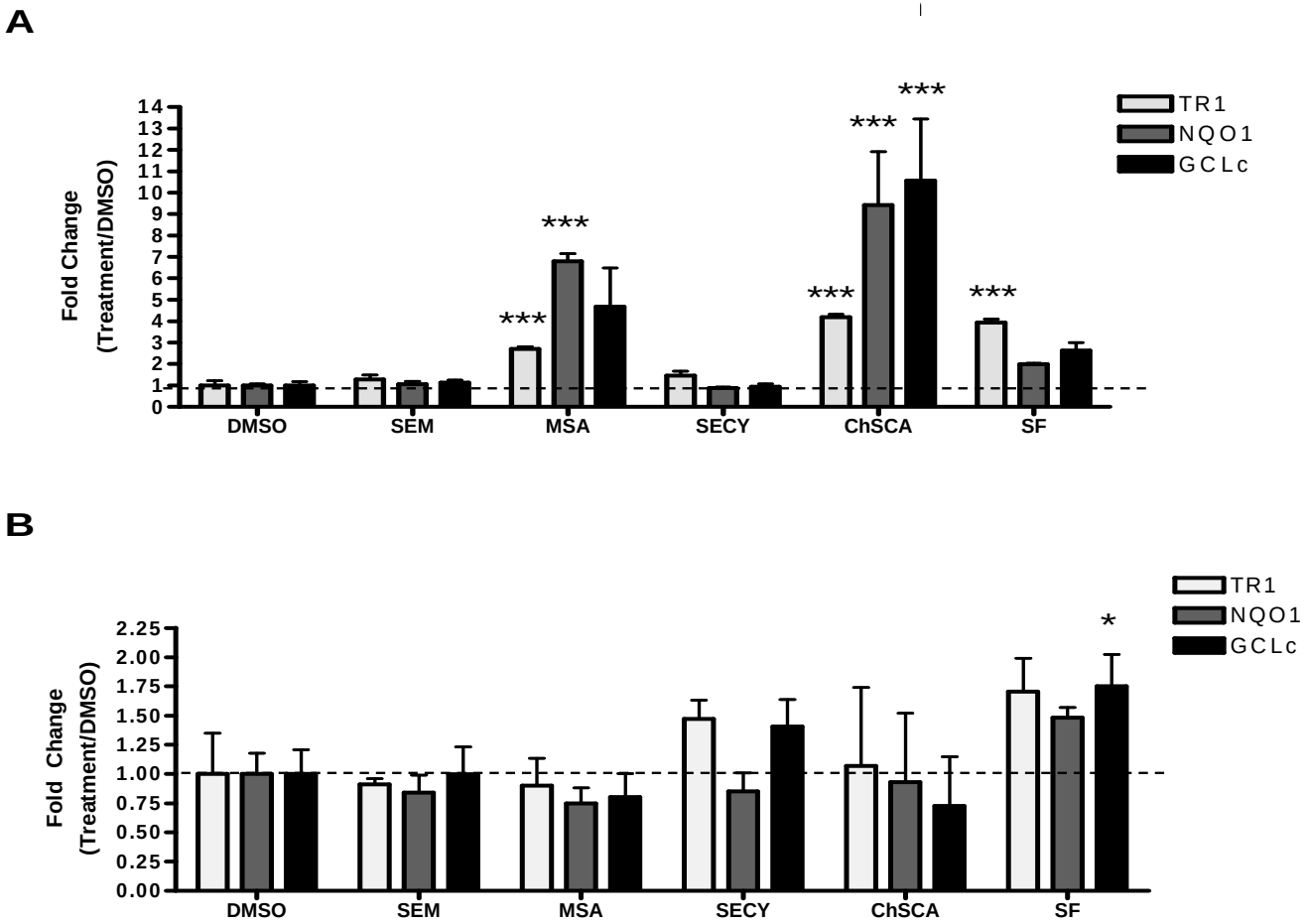

Figure 3.4. Induction of Nrf2 target genes by selenocompounds is lung cell line-specific. Data are presented as mean fold change relative to DMSO control. **(A)** mRNA expression in NHBE cells following 12 hour treatment. Treatment concentrations were 100 μM SEM, 2.5 μM MSA, 5 μM SECY, 5 μM ChSCA, and 1 μM SF. '***' indicates $p<0.001$ versus DMSO control **(B)** mRNA expression in A549 cells following 24 hour treatment. Treatment concentrations were 100 μM SEM, 2.5 μM MSA, 100 μM SECY, 50 μM ChSCA, and 10 μM SF. '*' indicates $p<0.05$ versus DMSO control.

sulforaphane, only significantly increased TR1 mRNA expression at the 12 hour time point.

In a human lung adenocarcinoma cell line in which the Nrf2/ARE induction pathway is constitutively active, no change in the mRNA expression of TR1, NQO1, or GCL_C was observed for any selenocompound (Figure 3.4B). Sulforaphane was the only compound to produce a significant induction in this A549 cell line, a 2-fold increase in GCL_C.

Effects of Selenocompounds on Redox Balance

Increased cellular oxidative tone is known to be one mechanism by which the Nrf2 induction pathway can be activated. We sought to determine whether selenocompound exposure elicits a change in oxidation that could lead to Nrf2 pathway activation in the BEAS-2B cells. All the selenocompounds and sulforaphane produced a rapid (within 2 hours) increase in reactive oxygen species (Figure 3.5A) and a decrease in intracellular glutathione content (Figure 3.5B). As with the 24 hour time point above (Figure 3.3B), changes in reduced GSH mirrored those for total GSH, indicating that the compounds are depleting GSH and not oxidizing GSH to GSSG. MSA was the least effective agent in depleting glutathione.

Effect of Selenocompounds on Nrf2 Nuclear Localization

In order for Nrf2 to drive transcription of ARE genes, it must translocate from the cytosol to the nucleus. We performed Western blotting of BEAS-2B cell nuclear lysates to determine if, after 4 hours, selenocompounds increased the amount of nuclear, and

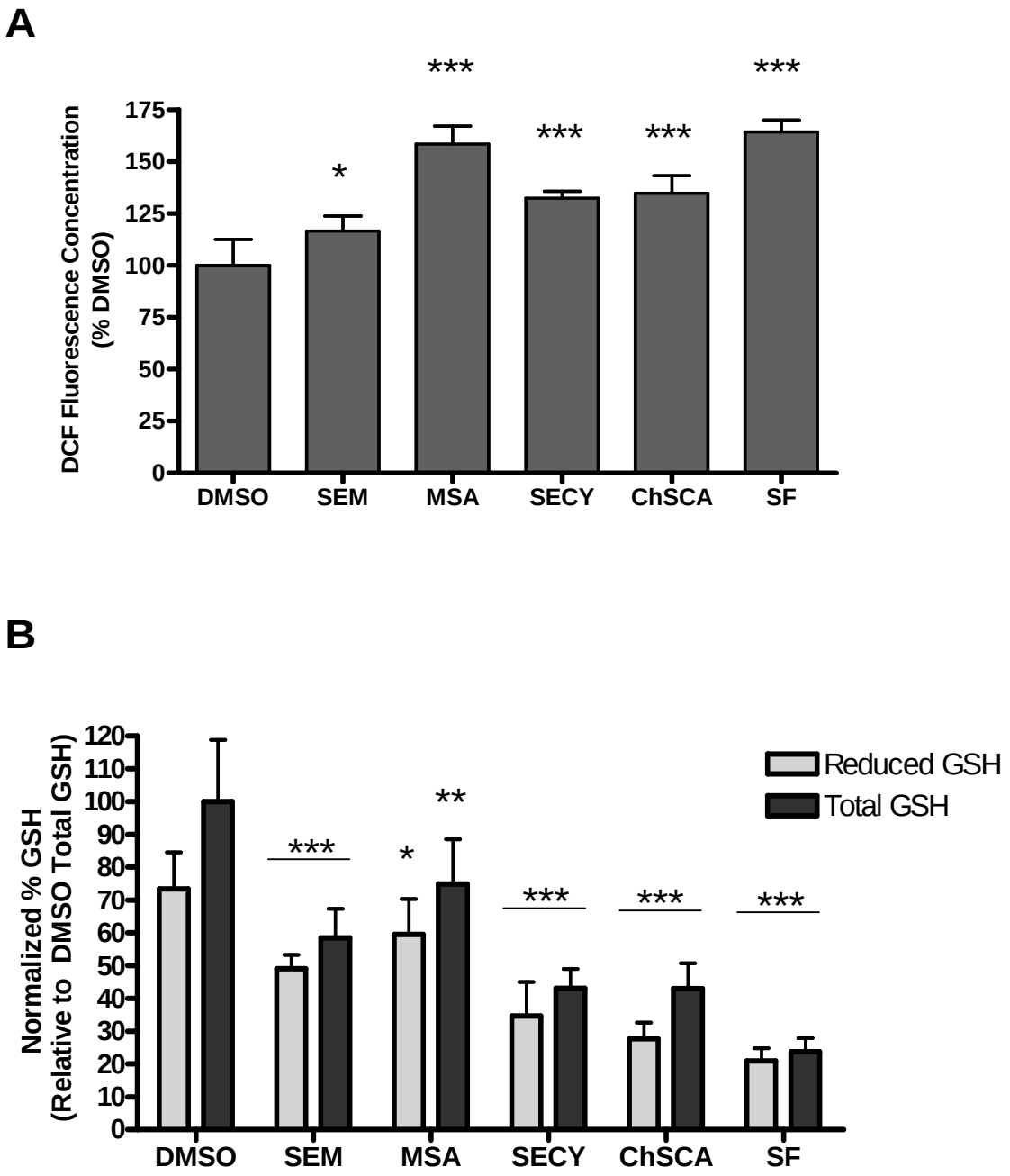

Figure 3.5. Effects of selenocompounds on ROS levels and intracellular GSH in BEAS-2B cells at 2 hours. Treatment concentrations were 100 μM SEM, 2.5 μM MSA, 1 μM SECY, 25 μM ChSCA, and 5 μM SF. **(A)** DCF fluorescence concentration. Data are presented as mean % DMSO control and are for the PI negative population. Significant differences are denoted by: '*' indicates $p<0.05$ and '***' indicates $p<0.001$ versus DMSO control, n=3 for all treatments.. **(B)** Reduced and total intracellular GSH content. Data are normalized to cell viability fluorescence measurements and are presented as mean % DMSO total glutathione. n=4 for all treatments. '*' indicates $p<0.05$, '**' indicates $p<0.01$ and '***' indicates $p<0.001$ versus respective DMSO control.

therefore active, Nrf2 protein. Nuclear Nrf2 was detectable in cells treated with DMSO, indicating that basal Nrf2 activity exists in BEAS-2B cells. Selenocystine, ChSCA, and MSA, but not selenomethionine, increased Nrf2 localization in the nucleus to an equal or greater extent than sulforaphane (Figure 3.6).

Nrf2 Knockdown Attenuates ARE Gene Induction by Selenocompounds

To determine if Nrf2 is responsible for the increased mRNA expression of ARE-responsive genes by selenocompounds, we used small interfering RNA (siRNA) to transiently depress Nrf2 expression. Low basal protein expression of Nrf2 in BEAS-2B cells necessitated stimulation with sulforaphane to assess protein attenuation with the use of siRNA. In cells transfected with control siRNA, a small amount of Nrf2 protein is expressed in DMSO vehicle treated cells but sulforaphane significantly increased Nrf2 protein expression. However, the induction of Nrf2 protein by sulforaphane was attenuated by greater than 95% in the cells transfected with Nrf2-specific siRNA (Figure 3.7A). To confirm that a functional knockdown was achieved, TR1 and NQO1 protein expression levels were also determined from the sulforaphane-treated cells. Both proteins show increased expression with sulforaphane stimulation in cells transfected with control siRNA, but the induction of TR1 and NQO1 following sulforaphane stimulation was not observed in the cells transfected with Nrf2-specific siRNA. GAPDH was used as a loading control and did not change with sulforaphane stimulation.

The mRNA levels of TR1, NQO1 and GCL_C were assessed following treatment with the siRNAs as well as the selenocompounds or sulforaphane. Nrf2 knockdown decreased basal TR1, NQO1, and GCL_C mRNA expression (Figure 3.7B, C, and D) to

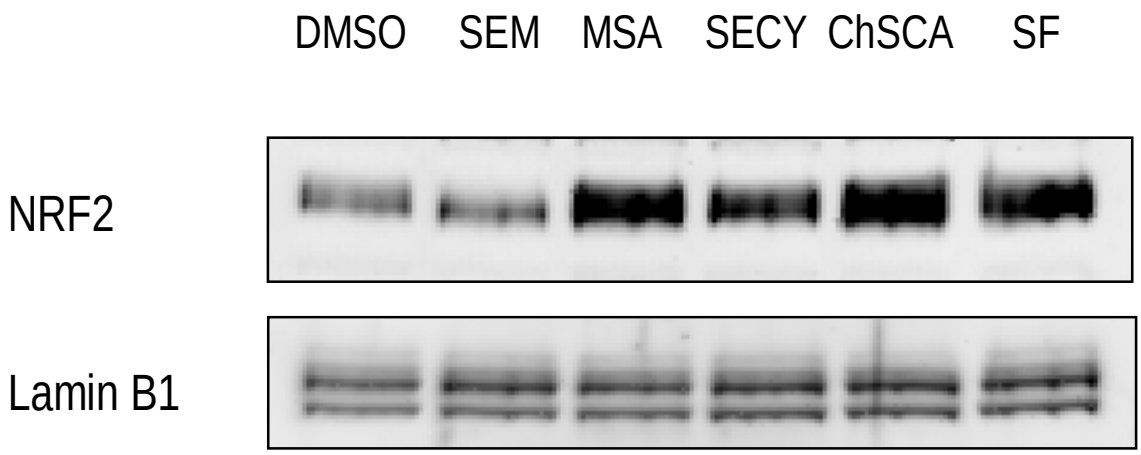

Figure 3.6. Selenocompounds increase Nrf2 nuclear protein expression. Immunoblotting of Nrf2 in BEAS-2B nuclear lysate fractions after 4 hour treatments. Treatment concentrations were 100 μM SEM, 2.5 μM MSA, 1 μM SECY, 25 μM ChSCA, and 5 μM SF. 20 μg nuclear lysate was loaded per sample. Lamin B1 served as nuclear protein loading control. The immunoblot shown is representative of two independent experiments.

0.35, 0.20, and 0.45 that of "control" siRNA cells, respectively. The expression of TR1, NQO1, and GCL_C in cells transfected with control siRNA (Figure 3.7B, C, and D) the same pattern as in nontransfected BEAS-2B cells (Figures 2A, 2B, and 3A). Selenocystine, ChSCA, MSA, and sulforaphane increased TR1 and NQO1 expression in "control" siRNA treated cells at 24 hours (Figure 3.7B and C). Selenocystine, ChSCA, and sulforaphane, but not MSA, increased GCL_C mRNA levels (Figure 3.7D). The increases in mRNA expression of TR1, NQO1, and GCL_C by selenocompounds or sulforaphane were all attenuated with Nrf2 knockdown.

Discussion

Lung cancer is the leading cancer type for cancer deaths. There were an estimated 222,520 new cases of lung and bronchus cancer in the United States in 2010, making it the second most common cancer type [28]. Although other cancer types have seen improvements in survival rates, the five-year survival rate for lung cancer has only improved marginally from 13% in 1975 to 16% in 2005. The poor prognosis associated with lung cancer can be attributed to the typical late stage diagnosis of the disease and failure of chemotherapy. Because of this poor clinical prognosis and the large population susceptible to lung cancer from tobacco smoke, novel strategies for lung cancer prevention are necessary.

A role for Nrf2 as a protective pathway in the lung has been well established over the past decade. Several studies using Nrf2 knockout mice have demonstrated the ability of Nrf2 to protect the lung from oxidative damage. Nrf2 knockout mice are more susceptible to developing airway diseases such as asthma [29], emphysema [30-32], and pulmonary fibrosis [33]. Lungs of Nrf2 knockout mice were more sensitive to cigarette

Figure 3.7: Attenuation of selenocompound-induced antioxidant gene expression with Nrf2 knockdown. BEAS-2B cells were transfected with Nrf2 or control siRNA for 24 hours and then treated with compounds for 24 hours. **(A)** Knockdown of Nrf2 and Nrf2 target genes as assessed by Western blot. Cells were treated with 1 μL/mL DMSO or 5 μM SF. 25 μg whole cell lysate proteins were loaded per sample. GAPDH was probed as loading control. The blot shown is representative of two independent experiments. **(B, C, D)** TR1, NQO1, and GCL_C mRNA expression in control and Nrf2 siRNA cells. Treatment concentrations were 2.5 μM MSA, 1 μM SECY, 25 μM ChSCA, and 5 μM SF. Data are presented as fold change relative to DMSO control siRNA. n=3 for all treatments. '**' indicates $p<0.01$ and '***' indicates $p<0.001$ versus DMSO control siRNA. '##' indicates $p<0.01$ and '###' indicates $p<0.001$ versus matched control siRNA treatment.

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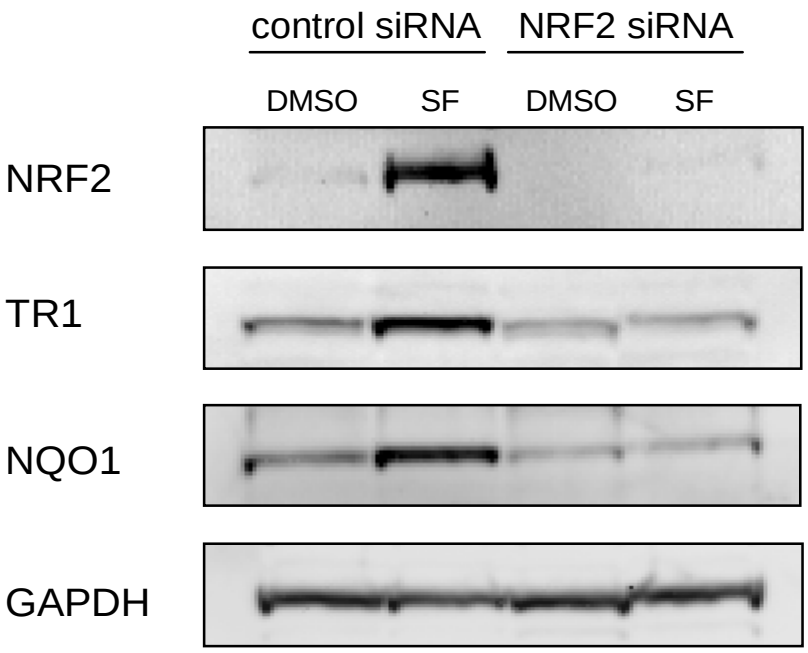

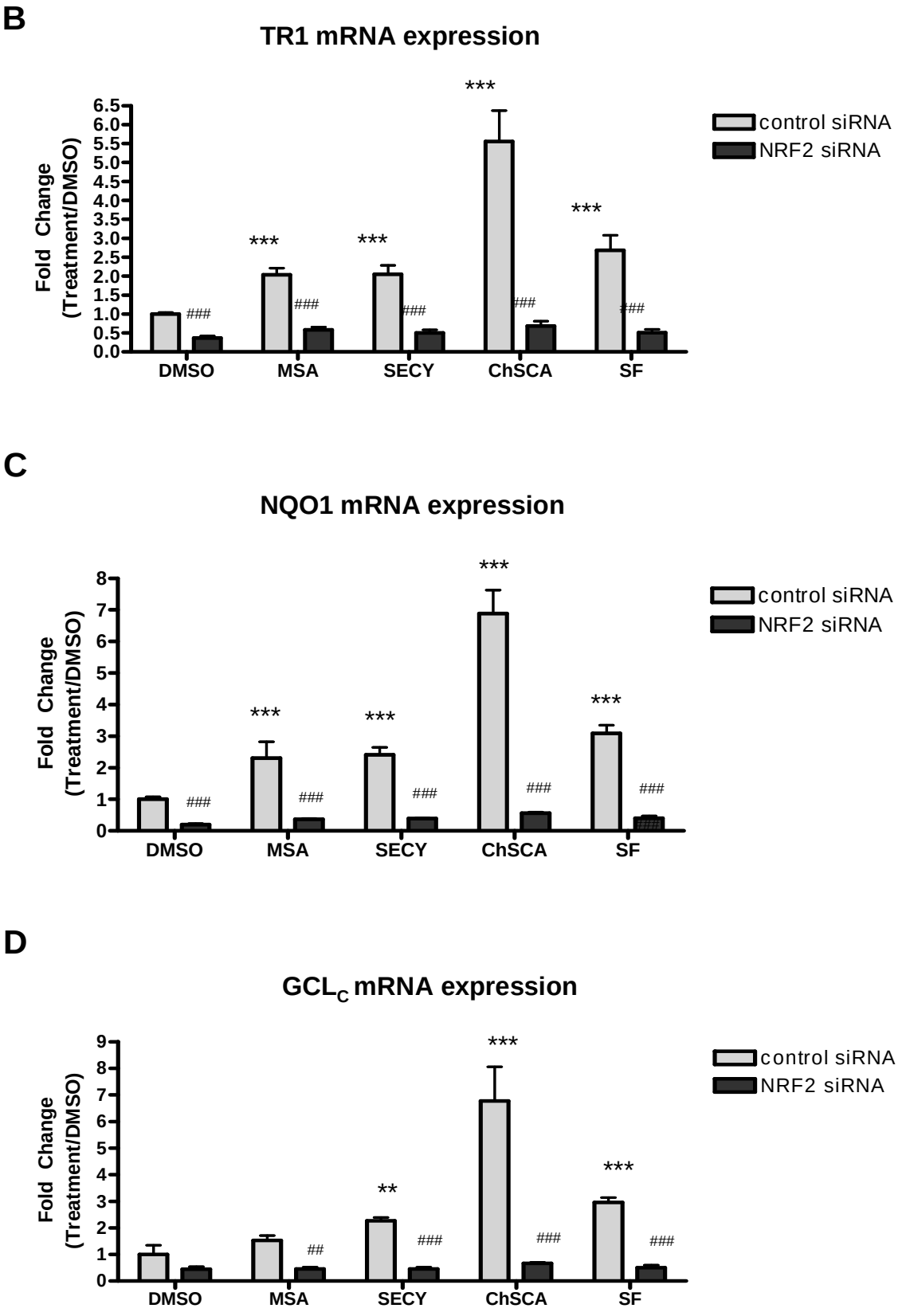

Figure 3.7 continued

smoke [30], butylated hydroxytoluene [34], diesel exhaust [35], and hypoxia [36], and developed more spontaneous and benzo(*a*)pyrene-induced genomic DNA mutations [37]. Lung epithelial cells isolated from $Nrf2^{-/-}$ mice also demonstrate enhanced sensitivity to oxidants in comparison to cells from $Nrf2^{+/+}$ mice [38]. These Nrf2 deficient lung cells have an altered redox balance, demonstrated by increased basal ROS and decreased basal GSH levels. Strategies that genetically enhance Nrf2 activity have further indicated a protective role of Nrf2 in the airway. Lung cells with decreased expression of the negative regulator Keap1 exhibit greater antioxidant capacity as demonstrated by increased expression of antioxidant and phase II genes and decreased ROS generated from H_2O_2 treatment [39]. Pharmacological treatments to increase Nrf2 activity in the lung have also demonstrated benefits [40, 41]. Studies in both mice and humans have shown Nrf2 activation and phase II gene induction in the airway in response to cigarette smoke [30; 42, 43], presumably to negate its oxidative effects. A protective role for Nrf2 in the susceptibility of several cancers has been shown [see 45 for a review], but has yet to be explicitly tested in a lung cancer model. Still, Nrf2 and phase II enzyme induction are recognized mechanisms of lung cancer prevention [6, 44].

Clinical selenium supplementation trials have demonstrated variable benefits for lung cancer. The NPC trial, which provided selenium as selenized yeast, did not find a significant benefit with selenium for its primary endpoint of nonmelanoma skin cancer, but did indicate a decrease in lung cancer incidence and mortality [5]. This benefit was diminished in the total population with extended follow-up, but remained significant in participants with low baseline plasma selenium levels [45]. SELECT was the largest supplementation trial to date, enrolling over 35,000 men [46].The primary endpoint of

SELECT was prostate cancer, but also analyzed lung cancer incidence as a secondary endpoint. Selenomethionine, the most prevalent selenocompound found in the selenized yeast used for the NPC trial, served as the selenium form. SELECT found no benefit of selenomethionine in regards to lung cancer incidence or all cancer mortality. However, the majority of the SELECT population had higher baseline selenium levels than that of the NPC trial population, indicating that selenium status may be an important factor in the chemopreventive effects of selenium. The different forms of selenium may also have contributed to these differential findings. A better understanding of selenium biology in cancer prevention, such as that taken from experiments in this study, is warranted, as well as investigation into which populations would most benefit from selenium [47, 48].

In our previous work, we have sought to identify novel selenocompounds that exhibit efficacy in lung cancer chemoprevention. Selenocysteine demonstrates intriguing properties for chemoprevention, but can be chemically unstable. Selenazolidines were designed to be selenocysteine delivery agents and patterned after thiazolidine sulfur compounds [49]. This group of compounds is comprised of selenazolidine-4(R)-carboxylic acids with varying 2-substituents. Many of these compounds, including ChSCA, are thought to release selenocysteine through nonenzymatic spontaneous hydrolysis. These compounds, along with the selenocysteine dimer selenocystine, have been assessed for chemoprevention efficacy in the NNK model of lung cancer [1, 2] with varying success.

With the animal study design used in this work, selenocystine treatment occurs in the post-initiation phase and more closely models the intervention strategy for a former smoker. Our previous study indicated that selenocystine is only effective at decreasing

lung tumors following NNK administration [2]. Using this approach, we show that selenocystine induces Nrf2-regulated ARE genes related to glutathione metabolism and oxidoreductase activity (Figure 3.1). We then utilized *in vitro* experiments in nonmalignant lung cell lines to further ascertain the mechanism of action of selenocompounds. These lines serve to model lung cells at the pre-initiation step of carcinogenesis where chemopreventive agents can be beneficial. Selenocystine and the other compounds investigated, ChSCA, MSA, and selenomethionine increased antioxidant genes in BEAS-2B cells (Figures 3.2 and 3.3A). ChSCA and MSA demonstrated similar ability in the NHBE cell line (Figure 3.4A). These findings indicated that selenocompound induction of antioxidant genes is not limited to the mouse. The observed increases in antioxidant gene expression at 24 hours were preceded by generation of ROS (Figure 3.5A), depletion of GSH (Figure 3.5B), and translocation of Nrf2 to the nucleus (Figure 3.6). This is indicative that the selenocompounds generate an oxidative insult to promote Nrf2 activation. Interestingly, *de novo* synthesis of GSH appears to be a component of the recovery from the insult rather than recycling of oxidized GSSG to reduced GSH. By 24 hours, GSH levels had returned to or were greater than baseline (Figure 3.3B). Lack of increased gene expression in Nrf2 siRNA knockdown cells further argues for an Nrf2 dependent mechanism of the selenocompounds (Figure 3.7). Our previous work has shown that the selenocompounds at the same or higher concentrations do not increase ROS levels in BEAS-2B cells at 24 hours [17], indicating that the increased levels at 2 hours are attenuated by 24 hours, potentially via Nrf2. Taken together, our data support a mechanism by which selenocompounds initially behave as pro-oxidants to activate the Nrf2 pathway, and it is

through this Nrf2 activation and subsequent ARE gene induction that they indirectly exert endogenous antioxidant effects.

Of the selenocompounds investigated, ChSCA produced the most robust response in regard to mRNA expression in both the BEAS-2B and NHBE cells (Figures 3.2, 3.3A, 3.4A). Selenocystine also increased ARE gene expression in mouse lung tissue (Figure 3.1) and BEAS-2B cells (Figures 3.2, 3.3A), but did not modulate ARE gene transcription in NHBE cells (Figure 3.4A). MSA increased TR1 and NQO1 expression in both the BEAS-2B and NHBE cells (Figures 3.2, 3.4A) with the magnitude of induction greater in the NHBEs. Unlike the other compounds investigated, MSA did not increase GCL_C mRNA expression (Figure 3.3A), but was able to increase BEAS-2B GSH levels to a similar extent as ChSCA and selenocystine (Figure 3.3B). These three compounds all increased intracellular GSH levels and nuclear Nrf2 protein expression to extents similar or greater than sulforaphane (Figures 3.3B, 3.6). Selenomethionine was able to increase ARE gene expression (Figures 3.2A, 2C, and 3.3A), but did not increase nuclear Nrf2 protein (Figure 3.6) or GSH levels (Figure 3.3B) in the BEAS-2B cells like the other compounds. That selenomethionine failed to increase either nuclear Nrf2 or GSH levels does not appear to be a concentration issue, as selenomethionine was used at a concentration four times greater than the next highest selenocompound concentration (25 μM ChSCA). The results from this study indicate that ChSCA, SECY, and MSA are the most effective at activating the Nrf2 pathway in the lung and inducing its downstream effects.

Previous reports on the mechanisms of selenocompounds in lung cells have not indicated changes in expression of antioxidant response genes, but rather cell cycle and

apoptotic genes. A microarray study with *p*-XSC showed changes in transcription factor, growth factor, and apoptotic gene expression [50], while MSA induced alterations in cell cycle genes involved in the G_1 to S phase transition, resulting in G_1 phase arrest [4]. Changes in apoptosis or cell cycle genes were not indicated in our microarray analysis of lung tissue from animals fed selenocystine (Figure 3.1). However, it must be pointed out that the model systems differ. Our results are for nontumorigenic mouse lung tissue and nonmalignant human lung cells. Human non-small cell lung cancer lines were utilized for the *p*-XSC and MSA gene expression studies (H460 and H520 cells, respectively). The effects of selenium seen in these malignant cells are likely to differ from what would be observed in nonmalignant cells. Many lung cancer cell lines that have progressed to the transformation step of carcinogenesis have Keap1 or Nrf2 mutations that result in constitutively active Nrf2 and high basal antioxidant gene expression [51, 52]. Thus, these cell lines may not serve as an appropriate system in which to observe an induction of antioxidant defense systems. This was evidenced by the lack of increased antioxidant and glutathione gene expression with selenocompounds we observed in A549 cells (Figure 3.4B). A549 cells harbor a KEAP1 mutation that results in the constitutive activation of Nrf2 [51]. Since Nrf2 pathway activation is already maximized in this cell line, it is not possible for the selenocompounds to further increase mRNA expression via Nrf2. This lack of selenocompound-induced increases in gene expression supports an Nrf2-mediated mechanism.

In comparison, primary NHBE cells responded most dramatically to MSA and ChSCA. The differential effects of the selenocompounds in NHBEs as compared to BEAS-2Bs may be due to cell population characteristics of the two lines. BEAS-2B cells

are derived from NHBE cells pooled from several noncancerous donors and transformed with a 12-SV40 adenovirus hybrid. It is unknown if the NHBE cells exhibit a pathology that would cause them to be less responsive to the antioxidant inducer sulforaphane. Differences in cell culture medium for the two lines may also contribute to the differential patterns of induction. Since a similar induction of antioxidant genes was observed with selenocystine in BEAS-2B cells as the mouse lung, we utilized this cell line for further experiments related to the mechanism of enzyme induction.

Other selenocompounds have been shown to be inducers of phase II enzyme expression and activity. Glutathione S-transferase and total GPx activity was increased by one week of dietary *p*-XSC in mouse lung and liver, but no change in selenium-dependent GPx or UGT activity was found [53]. *p*-XSC has also been shown to increase GSH and antioxidant levels in the lung of A/J mice [54]. It is unknown whether *p*-XSC acts through an Nrf2 mechanism. Various other selenocompounds have also induced antioxidant and phase II enzymes in tissues other than the lung [55-58]. A recent study evaluating 3-selena-1-dethiacephem compounds in LNCaP prostate cancer cells suggested that the mechanism of action of two of these compounds included Nrf2 activation [59].

The mechanism by which the selenocompounds in this study activate Nrf2 remains to be elucidated. One potential mechanism is modification of critical Keap1 cysteine residues to activate Nrf2. Sulforaphane has been shown to disrupt the Nrf2-Keap1 complex by forming thionoacyl adducts with Keap1 [60]. In contrast, other electrophiles, such as tert-butylhydroquinone, allow for the alkylation of Keap1 and its subsequent polyubiquitination [61]. Whether selenocompounds or their metabolites

directly disrupt the Nrf2-Keap1 complex remains to be determined. ROS generation and GSH depletion (Figure 3.5) may also be involved in Nrf2 activation. Moderate GSH depletion (~40%) is insufficient to activate Nrf2 and it is not until levels reach 10-15% of baseline that Nrf2 is activated by this depletion alone [62, 63]. At 2 hours, the selenocompounds depleted GSH but not to an extent that would be expected to activate Nrf2 alone (Figure 3.5B). Further, Nrf2 activation does not require GSH depletion, as compounds such dexamethasone 21-mesylate and 15-deoxy-Δ-$^{(12,14)}$-prostaglandin J_2 do not decrease GSH levels, but are able to activate Nrf2 by directly interacting with Keap1 [63]. Therefore, it is unlikely that GSH depletion is the primary mechanism. Other indirect mechanisms of Nrf2 activation may also be involved. MSA was shown to inhibit glycogen synthase kinase 3 beta [64], which can in turn lead to nuclear Nrf2 accumulation [65].

In conclusion, we demonstrate that selenocompounds increase antioxidant and glutathione genes in the lung, and do so through in an Nrf2-dependent mechanism. This finding will be of use in further evaluating the chemopreventive mechanisms of selenocompounds and their use in the lung.

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CHAPTER 4

THIOREDOXIN REDUCTASE 1 KNOCKDOWN ENHANCES SELENAZOLIDINE CYTOTOXICITY IN HUMAN LUNG CANCER CELLS VIA MITOCHONDRIAL DYSFUNCTION

Abstract

TR1 is a selenoprotein that is involved in cellular redox status control and deoxyribonucleotide biosynthesis. Many cancers, including lung, overexpress TR1, making it a potential cancer therapy target. Previous work has shown that TR1 knockdown enhances the sensitivity of cancer cells to anticancer treatments, as well as certain selenocompounds. However, it is unknown if TR1 knockdown produces similar effect on the sensitivity of human lung cancer cells. To further elucidate the role of TR1 in the mechanism of selenocompounds in lung cancer, a lentiviral microRNA delivery system to knockdown TR1 expression in A549 human lung adenocarcinoma cells was utilized. Cell viability was assessed after 48 hr treatment with the selenocysteine prodrug selenazolidines BSCA and ChSCA, selenocystine, MSA, *p*-XSC, and selenomethionine. TR1 knockdown increased the cytotoxicity of BSCA, ChSCA, and selenocystine but did not sensitize cells to MSA, selenomethionine, or *p*-XSC. GSH and TR1 depletion together decreased cell viability, while no change was observed with GSH depletion alone. ROS generation was induced only in TR1 knockdown cells treated with the

selenazolidines or selenocystine. These three compounds also decreased total intracellular glutathione levels and oxidized thioredoxin, but in a TR1 independent manner. TR1 knockdown increased selenazolidine and selenocystine-induced mitochondrial membrane depolarization, as well as DNA strand breaks and apoptosis inducing factor translocation from the mitochondria. These results indicate the ability of TR1 to modulate the cytotoxic effects of BSCA, ChSCA and selenocystine in human lung cancer cells through mitochondrial dysfunction.

Introduction

TR is a selenoprotein that functions to reduce the oxidoreductase Trx in a NADPH-dependent manner. Together, this thioredoxin system is an important regulator of cellular redox status. TR is also involved in cell proliferation, DNA replication, cell cycle, transcription factor regulation, and other redox-sensitive cell signaling pathways [1-3]. TR contains selenium in the form of selenocysteine as the penultimate residue at the C-terminus. In humans, TR is found in all tissues and is expressed as two major isoforms: cytosolic (TR1) and mitochondrial.

Many cancers, NSCLC, have high expression levels of both TR1 and Trx. In the lung, TR and Trx expression correlate with cell proliferation, survival, and prognostic factors such as lymph node status and tumor grade [4-6]. TR1 knockdown reversed the malignant phenotype and decreased tumor growth and metastasis of murine lung carcinoma cells further implicating TR1 in cancer development [7]. TR1 was also shown to be involved in the tumor phenotype of malignant cells [1]. Overexpression of the Trx system has been implicated in cell resistance to oxidative and electrophilic insults,

including some selenocompounds [8-13]. Thus, TR can protect tumor cells from the oxidative stress generated by many anticancer drugs. These findings indicate a role for the TR system in lung cancer development, progression, and chemoresistance, and identify it as a potential therapeutic target.

Selenium supplementation trials have had mixed results in regard to cancer incidence and mortality [14-17], but the use of selenium in potential cancer treatment remains of interest. Several selenocompounds have demonstrated anticancer activity in cell culture and *in vivo* studies. MSA induces cell cycle arrest and apoptosis in lung cancer cells [18] and is effective at inhibiting xenograft growth [18, 19]. The organoselenocompound *p*-XSC has also demonstrated anticancer activity in lung cancer models where selenomethionine was not effective [20-22]. Selenocystine and two selenazolidine compounds that were designed to nonenzymatically release selenocysteine also decreased murine lung tumors when administered post-initiation [23], but did not affect transcriptional levels of cell cycle or apoptotic genes. Together, these studies indicate that certain selenocompounds exhibit anticancer properties in the lung, though potentially via differential mechanisms that require elucidation.

Since overexpression of the Trx system contributes to the high antioxidant capacity of NSCLCs, we sought to determine if decreasing TR1 would increase the sensitivity of malignant cells to redox-modulatory selenocompounds. Specifically, the aim of this work was to determine if knockdown of TR1 in A549 human NSCLC cells would affect the cytotoxicity and redox effects of several chemically distinct organoselenocompounds: two selenocysteine prodrug selenazolidines, BSCA and ChSCA, selenocystine, MSA, and selenomethionine. A549 cells express some of the

highest levels of TR1 as well as other antioxidant genes due to a KEAP1 mutation [24], and therefore, present a unique system to determine role of TR1 in the context of cells with many antioxidant genes overexpressed. For comparison, we also utilized the H1666 human NSCLC cell line, which has lower basal TR1 expression than the A549 cell line and does not have a known KEAP1 mutation [24].

Materials and Methods

Reagents

The A549 adenocarcinoma cell line (A549 ATCC) was purchased from American Tissue Type Culture Collection (Manassas, VA). The H1666 adenocarcinoma cell line was a kind gift from Dr. Andrea Bild (University of Utah). BSCA and ChSCA were synthesized as described [25]. L-selenomethionine was from Acros Organics (Morris Plains, NJ), MSA was from PharmaSe, Inc. (Lubbock, TX), and *p*-XSC was from LKT Laboratories, Inc (St. Paul, MN). L-selenocystine, DMSO, DMF, CDDP, NAC, tiron, recombinant thioredoxin from *E. coli*, insulin solution from bovine pancreas, iodoacetic acid, iodoacetamide, 40% acrylamide/bis-acrylamide 37.5:1 solution, and ammonium persulfate were purchased from Sigma-Aldrich (St. Louis, MO). Advanced Dulbecco's modified Eagle's medium (DMEM), Glutamax, pcDNA™6.2-GW/EmGFP-miR, pLenti4/TO/DEST and pLenti6/TR vectors, blasticidin S HCl, Zeocin™, tetracycline, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), propidium iodide (PI), MitoProbe JC-1 assay kit, NuPAGE Bis-Tris gels, APO-BrdU TUNEL assay kit, and bovine serum albumin were from Invitrogen (Carlsbad, CA). CellTiter-Glo Luminescent Cell Viability,

MultiTox-Fluor Multiplex Cytotoxicity, GSH-Glo Glutathione, and Caspase-Glo 3/7 assays were purchased from Promega Scientific (Madison, WI). Fetal bovine serum was from HyClone (Logan, UT). Mitochondrial isolation kit for cultured cells, bovine serum albumin standard, Coomassie Plus Protein Reagent, SuperSignal West Dura extended duration substrate, and tris(2-carboxyethyl)phosphine hydrochloride (TCEP HCl) were from Thermo Scientific (Rockford, IL). Primary antibodies directed against TR1, Trx1, AIF, and GAPDH, and polyclonal horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); the α-tubulin primary antibody was from Zymed Laboratories (San Francisco, CA). Protease inhibitor cocktail tablets (complete) were from Roche (Indianapolis, IN) and PVDF membrane was from Millipore (Burlington, MA). Western Lighting chemiluminescence reagents were from PerkinElmer Life Sciences (Boston, MA). Nuclear Isolation and Staining Solution (NIM-DAPI) was from Beckman Coulter (Miami, FL). L-buthionine-(S,R)-sulfoximine (BSO) was purchased from Chemical Dynamics Corporation (South Plainfield, New Jersey). JNK inhibitor VIII and p38 MAP kinase inhibitor SB203580 were purchased from Calbiochem (La Jolla, CA) and Upstate Biotechnology (Lake Placid, NY), respectively. The HA-ASK1-wt and HA-ASK1-KM constructs were a kind gift from Dr. Hidenori Ichijo (Tokyo, Japan). The caspase inhibitor, Z-Asp-CH_2-DCB, was from Peptides International (Louisville, KY). Other common reagents were from Sigma-Aldrich (St. Louis, MO), Fisher Scientific (Pittsburgh, PA) or VWR Scientific (West Chester, PA).

Cell Culture

A549 and H1666 cells were cultured in advanced DMEM supplemented with 2% fetal bovine serum and 1% Glutamax. When supplemented with serum, advanced DMEM contains ~37 nM selenium, primarily in the form of sodium selenite. Cells were maintained at 37^{o}C in a humidified atmosphere of 5% CO_2. Selenocystine and selenomethionine were dissolved in advanced DMEM. MSA and BSO were dissolved in PBS and CDDP in DMF. All other compounds were dissolved in DMSO. Media was refreshed at the time of treatment and 0.01% DMSO added to MSA, selenomethionine and selenocystine-treated cells to maintain a constant DMSO concentration amongst treatments. For all experiments other than cell viability, compounds were used at the following concentrations: selenomethionine, 20 μM; MSA, 1 μM; selenocystine, BSCA, and ChSCA, 5 μM; CDDP, 20 μM.

Generation of Lentiviral miRNA Cell Lines

MicroRNA (miRNA) targeted against the TR1 mRNA sequence (NM_003330) beginning at nucleotide 752 was utilized to knockdown TR1 expression (‘miR-TR1’). miRNA targeted at a noneukaryotic gene (‘miNeg’) was used as a nonknockdown control. Both miRNAs were cloned into pcDNA™6.2-GW/EmGFP-miR and then cloned into the pLenti4/TO/DEST Gateway vector. A549 cells were transduced with either pLenti4/TO/EmGFP/miR-TR1 or pLenti4/TO/EmGFP/miNeg as well as pLenti6/TR to express tetracycline (tet) repression, creating a tet-inducible miRNA expression system. Stably transduced cells were selected for using Zeocin™ (pLenti4) and blasticidin (pLenti6). Fluorescence-activated cell sorting was used to obtain an enriched EmGFP

positive cell population, producing a population with a high percentage of virally transduced cells. Cells were maintained in advanced DMEM.1 μg/mL tet was added to culture medium at least 72 hours prior to seeding to induce miRNA expression.

Measurement of TR Activity by NADPH Oxidation

Whole cell lysates were collected in lysis buffer. Activity values were measured in duplicate and calculated using the slope of NADPH absorbance at 340 nm at 25^{o}C over a minimum of 30 minutes with a Perkin Victor V^{3} microplate reader as previously described [26].

Cell Viability by ATP Measurement

Cells were seeded into 384-well plates at a density of ~1.25 x 10^{3} cells/well in advanced DMEM and allowed to recover. Drug concentrations ranging from 0–60 μM (MSA, *p*-XSC), 0–100 μM (CDDP), 0–120 μM (SECY), or 0–600 μM (SEM, BSCA, ChSCA) were added in advanced DMEM and incubated with cells for 24, 48, or 72 hours. For BSO treatments, 20 μM BSO was added to cells at the time of seeding and refreshed every 24 hours. For antioxidant treatments, 5 mM NAC or 1 mM tiron was added to cells 2 hour prior to seeding. Viability was measured using CellTiter-Glo luminescent reagents as per the manufacturer's instructions. Luminescence was measured using the microplate reader.

Cell Clonogenic Assay

Cells were seeded into 6-well plates at a density of 300 cells/well in advanced DMEM. Cells were treated with drug concentrations ranging from 0-10 μM (SECY) or 0-20 μM (BSCA, ChSCA, MSA) for 7 days. Colonies were stained with MTT for 4 hours at 37°C. MTT concentrations were determined by solubilizing the MTT in 24:1 propanol:HCl and measuring the absorbance at 405 nm using the microplate reader.

Cell Cycle Analysis

After 48 hr treatments, cells were trypsinized, washed with PBS, resuspended in NIM-DAPI, and incubated for 1 hr in the dark. Samples were analyzed using flow cytometry (Cell Lab Quanta SC, Beckman Coulter) with a minimum of 20,000 events recorded for each sample. Cell cycle distributions were estimated using ModFit LT software Version 2.0.

Immunoblot Analysis

For the evaluation of TR1, whole cell lysates were collected as described [27] and protein concentrations determined by the Bradford method. Whole cell lysate was separated by a NuPAGE 10% Bis-Tris gel and transferred to a PVDF membrane. The membrane was blocked in 5% milk in TBS-T, probed with 1:200 anti-TR1 (B-2) monoclonal primary antibody, washed three times with TBST, and probed with 1:5000 donkey anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies. For Trx redox status, urea-PAGE and immunoblotting were performed as described [28, 29]. For the evaluation of apoptosis inducing factor (AIF), mitochondrial lysates were isolated

from cells using the Mitochondria Isolation Kit for cultured cells with Dounce homogenization protocol. Lysates were sonicated, clarified, and protein concentrations were determined using the Bradford method. Protein was separated by NuPAGE 4-12% Bis-Tris gels, transferred to PVDF membranes and membranes incubated with 1:1000 anti-AIF (H-300) polyclonal primary antibody, washed, and incubated with 1:5000 donkey anti-rabbit HRP-conjugated primary antibody. Protein was detected using chemiluminescence and visualized on a Kodak ImageStation 440.

Reactive Oxygen Species

Cells were incubated with 20 μM H_2DCFDA for 20 minutes at 37^{o}C following 48 hr treatment. Dichlorofluorescein (DCF) and PI fluorescence concentrations were cytometrically determined using a Beckman Coulter Cell Lab Quanta SC flow cytometer as described [27].

Determination of Intracellular Glutathione

MultiTox-Fluor reagent was added to wells following 48 hour drug treatments and incubated at 37^{o}C for 30 minutes. Live-cell fluorescence was measured at 505 nm using the microplate reader. Media was then removed from wells and GSH-Glo reagent containing 1 mM TCEP HCl was added. After incubation at 37^{o}C for 30 minutes, Luciferin Detection reagent was added to each well and incubated for 15 minutes at 37^{o}C. Luminescence was measured with the microplate reader. GSH concentrations were calculated from a GSH standard curve and normalized by the MultiTox-Fluor cell viability fluorescence values.

Cell Death Assays

Mitochondrial membrane depolarization was assessed using the JC-1 cytometric assay as described [27]. Caspase activity was measured as previously described [28] and luminescence was measured using the microplate reader. DNA fragmentation was measured using the APO-BrdU TUNEL assay kit. Cells were trypsinized, incubated in 1% (w/v) formaldehyde in PBS on ice for 15 minutes, washed with PBS, resuspended in cold 70% (v/v) ethanol and placed at -20^{o}C overnight. Cells were resuspended in 1 mL wash buffer and centrifuged for 5 minutes at 250 x *g*. Pellets were resuspended in a DNA-labeling solution containing terminal deoxynucleotidyl transferase and BrdUTP and incubated for 90 minutes at 37^{o}C. Cells were then washed and incubated with 100 μL Alexa Fluor 488 dye-labeled anti-BrdU antibody staining solution for 30 minutes at room temperature in the dark, followed by incubation with 0.5 mL PI/RNase A staining buffer. BrdU and PI fluorescence concentrations were measured at 525 nm and 670 nm, respectively, by flow cytometry.

Statistical Analysis

Data are presented as mean ± standard deviation. One-way ANOVA with Bonferroni post-hoc testing was used to determine statistical significance (GraphPad InStat Version 3.06). $p<0.05$ was considered significant. Two-way ANOVA with Holm-Sidak post-hoc analysis was used to evaluate the cell cycle analysis (SigmaStat Version 3.5).

Results

TR1 Knockdown in A549 Cells

TR1 protein expression was decreased by 95% in A549 miR-TR1 cells compared to A549 miNeg cells (Figure 4.1A). TR1 knockdown was further confirmed by measuring TR activity via an insulin-dependent NADPH oxidation assay. For A549 miR-TR1 cells, only ~20% TR activity remained (Figure 4.1B). The mitochondrial isoform likely contributes to the majority of the remaining TR activity in the miR-TR1 cells, as the NAPDH oxidation assay is not specific for the TR1 isoform. TR activity of A549 miNeg cells did not differ from nontransduced A549 cells ('A549 wt'). No observable differences in cell viability, morphology, or phenotype existed between the miNeg and miR-TR1 cell lines (Figure 4.1C).

Effects of TR1 Knockdown on Drug Cytotoxicity

Minimal differences in cell viability between A549 miNeg and miR-TR1 cells were observed with 24 hr drug treatments (data not shown). When treatments were carried out for 48 hours, decreases in A549 miR-TR1 cell viability were observed with the selenocysteine prodrug selenazolidines BSCA and ChSCA at concentrations of 5–100 μM (Figure 4.2) and selenocystine at concentrations of 1–10 μM. These compounds demonstrated >4-fold increases in sensitivity in miR-TR1 cells compared to miNeg cells. We also assessed the toxicity of the methylselenol precursor methylseleninc acid (MSA) and the selenoamino acid selenomethionine, but did not find differential toxicity with TR1 knockdown using treatment times up to 72 hours (72 hour data not shown). This lack of TR1-dependent sensitivity with selenomethionine in A549 cells

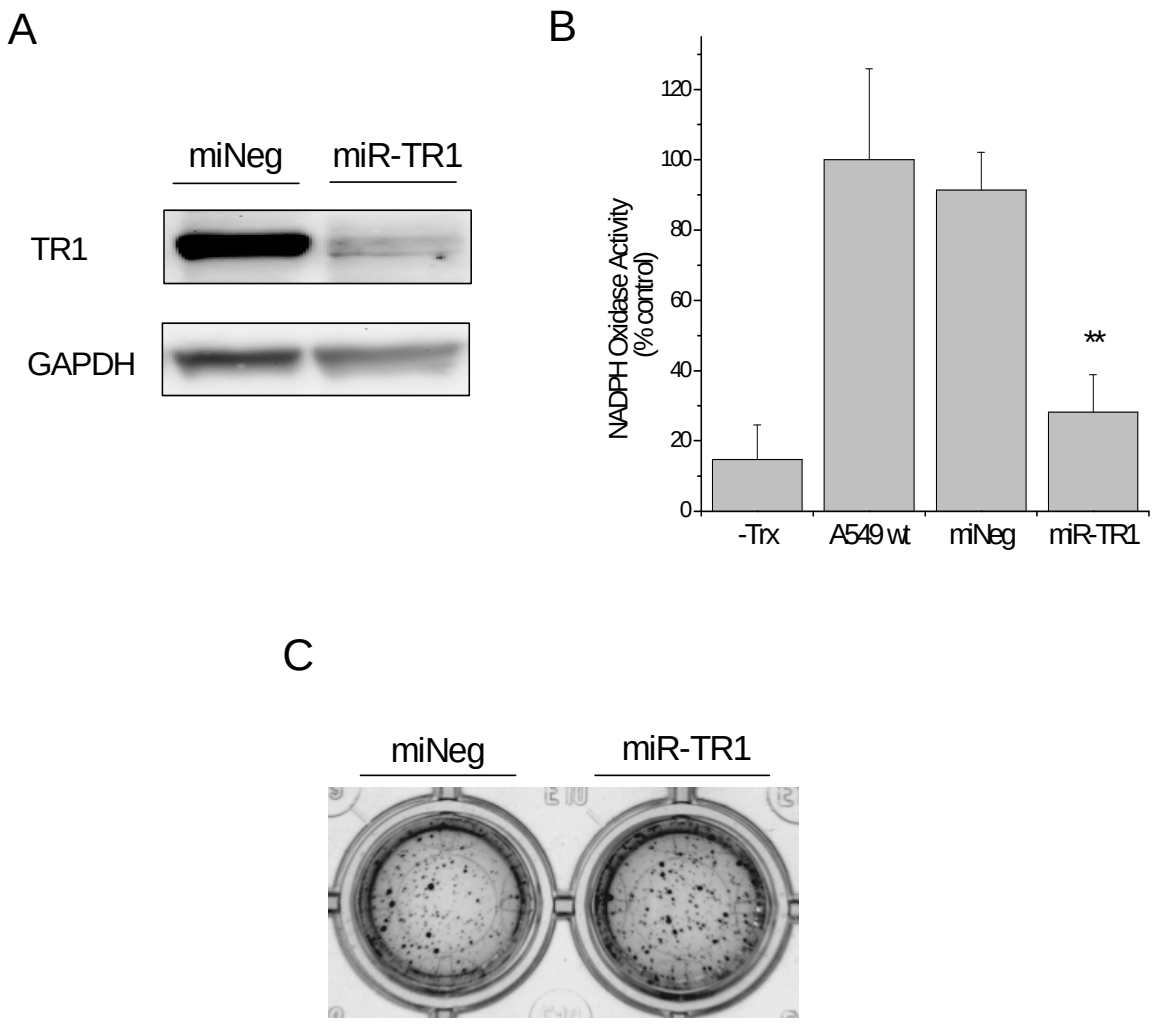
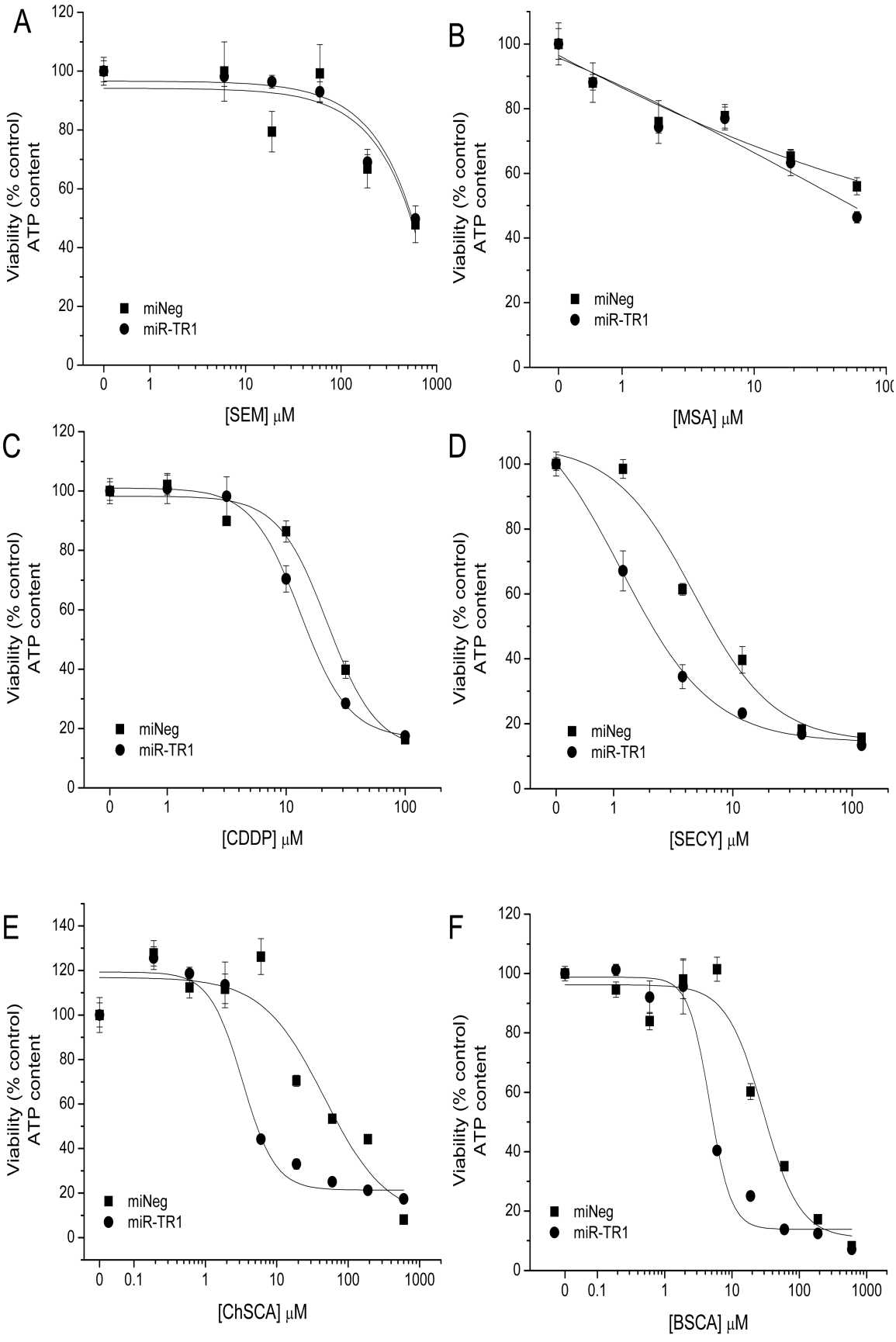


Figure 4.1. Knockdown of TR1 in A549 miR-TR1 cells by miRNA. A549 cells were transduced with lentiviral miRNA constructs to produce miNeg and miR-TR1 cell lines (see Materials and Methods). Cells were treated with 1 μg/mL tetracycline for 72 hours to induce miRNA expression. **(A)** Determination of TR1 protein expression by Western blotting. TR1 is the top band of the doublet. GAPDH was probed as a loading control using 1:500 anti-GAPDH polyclonal primary antibody. A similar decrease in TR1 protein expression was observed in A549 miR-TR1 cells up to 72 hours after removal of 1 μg/mL tetracycline. The blot shown is representative of two individual experiments. **(B)** Measurement of TR activity via NADPH oxidation. Data are presented as % A549 wt activity. n=2 for all groups. '*' indicates $p<0.05$ versus miNeg. NADPH oxidative activity in A549 cell lysates without added Trx (-Trx) in the reaction mixture, basal activity, activity in miNeg cells, and in the miR-TR1 cells. C) Anchorage independent growth assay. Cells were allowed to grow in 2% growth factor reduced Matrigel (BD Biosciences; Bedford, MA) and advanced DMEM supplemented with 1 μg/mL tet for 7 days, with media and tet refreshed every 3 days. Colonies were stained with MTT overnight and visualized on the Kodak ImageStation. No difference in colony growth was observed between the two cell lines. The data shown are representative of 6 replicates.

Figure 4.2. Effects of TR1 knockdown on drug cytotoxicity in A549 cells. Viability of A549 miNeg (■) and miR-TR1 (●) cells was assessed by measuring cellular ATP content after 48 hr drug treatment at the indicated concentrations. Viability values are expressed as % relative to vehicle controls, which were set to 100%. n=4 for all data points. No sensitization was observed in cells treated with **(A)** SEM or **(B)** MSA. Sensitization was observed in cells treated with **(C)** CDDP (miNeg IC_{50}: 22 ± 4 μM; miR-TR1 IC_{50}: 13 ± 0.5), **(D)** SECY (miNeg IC_{50}: 5 ± 1.4 μM; miR-TR1 IC_{50}: 1.2 ± 0.3), **(E)** ChSCA (miNeg IC_{50}: 48 ± 8 μM; miR-TR1 IC_{50}: 3 ± 1.1), and **(F)** BSCA (miNeg IC_{50}: 28 ± 8 μM; miR-TR1 IC_{50}: 5 ± 0.7).


A
Viability (% control)
ATP content
miNeg
miR-TR1
[SEM] μM
B
Viability (% control)
ATP content
miNeg
miR-TR1
[MSA] μM
C
Viability (% control)
ATP content
miNeg
miR-TR1
[CDDP] μM
D
Viability (% control)
ATP content
miNeg
miR-TR1
[SECY] μM
E
Viability (% control)
ATP content
miNeg
miR-TR1
[ChSCA] μM
F
Viability (% control)
ATP content
miNeg
miR-TR1
[BSCA] μM

is consistent with effects observed with these compounds in colon cancer cells [12]. We also determined if TR1 knockdown enhanced the toxicity of *cis*-platinum(II) diammine dichloride (CDDP, cisplatin), as it is a first-line treatment for NSCLC and interacts with TR1. A549 miR-TR1 cells were less than 2-fold more sensitive to CDDP after 48 hr treatment compared to A549 miNeg cells. These viability data suggest that TR1 can protect against selenium toxicity in a compound and cell line dependent manner.

To determine if the observed decreases in ATP content were a consequence of decreased cell proliferation, we conducted a clonogenic survival assay. Colony formation was observed in the miNeg and miR-TR1 cell lines after 7 days of treatment with MSA, selenocystine, BSCA, or ChSCA. All of these selenocompounds decreased clonogenic survival in a dose dependent fashion; however, replicate experiments did not demonstrate a statistically significant difference between miNeg and miR-TR1 cells. Representative examples of clonogenic survival are displayed in Figure 4.3. Taking these data into account with the ATP content viability data supports a mechanism whereby TR1 knockdown enhances selenocystine and selenazolidine cytotoxicity, but may not increase the sensitivity of A549 cells to these compounds in longer term experiments.

To further evaluate the role of these compounds in cell proliferation, the effects of TR1 knockdown and the selenocompounds that demonstrated a TR1-dependence in the ATP assay were evaluated for alterations in cell cycle population distributions. The effects of TR1 attenuation and selenocompound treatment were statistically significant but represent small shifts in the overall population (Table 4.1). Still, attenuation of TR1 alone decreased cells in G_1 phase and increased the population in S phase. Selenocystine

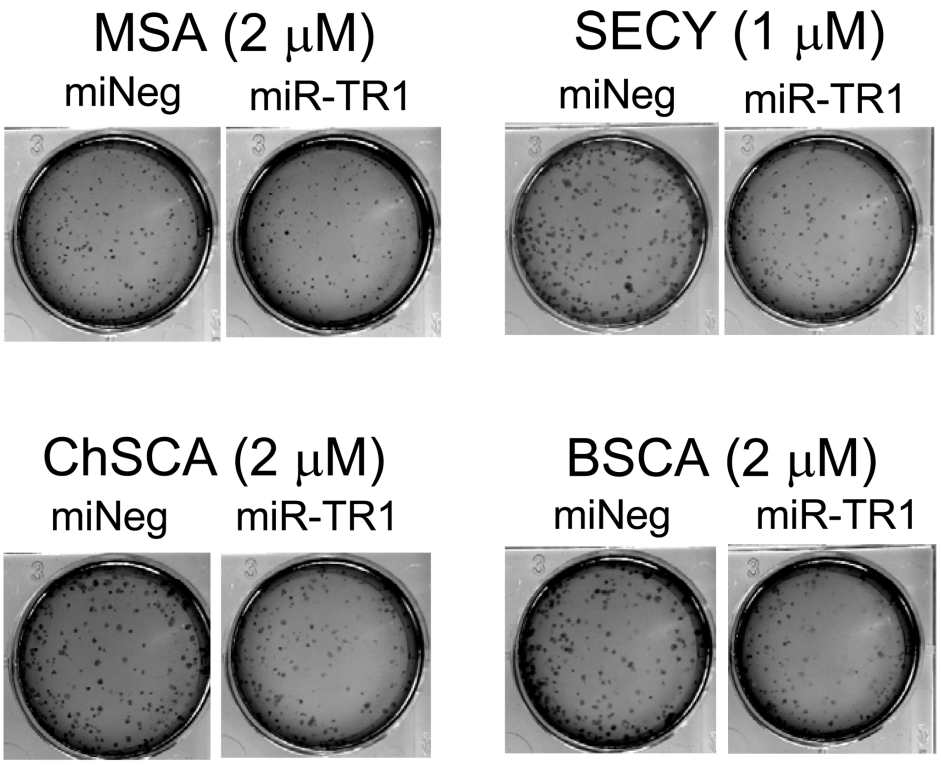


Figure 4.3. TR1 knockdown did not increase sensitization in clonogenic survival in A549 cells. Representative images of 7-day selenocompound treatment of A549 cells stained by the addition of MTT to the media and then visualized. Measurements of formazan-converted MTT indicate that statistically significant differences were not obtained between the miNeg and miR-TR1 cells.

Table 4.1: Cell Cycle Distribution

miNeg		**G_1**	**S**	**G_2/M**
	DMSO	68.0 ± 0.2	24.1 ± 0.6	7.9 ± 0.7
	SECY	75.5 ± 1.4[a]	18.8 ± 0.6[a]	5.8 ± 1.0
	BSCA	73.1 ± 2.0[a]	20.2 ± 1.3[a]	6.7 ± 1.6
	ChSCA	78.3 ± 0.7[a]	18.3 ± 0.9[a]	3.4 ± 1.3[a]
miR-TR1		**G_1**	**S**	**G_2/M**
	DMSO	64.2 ± 1.5[b]	27 ± 1.0[b]	8.8 ± 1.0
	SECY	69.9 ± 0.5[a,b]	23 ± 1.1[a,b]	7.1 ± 0.7
	BSCA	67.7 ± 0.9[a,b]	25.6 ± 1.2[b]	6.7 ± 0.2
	ChSCA	72.3 ± 2.2[a,b]	22.8 ± 2.0[a,b]	4.8 ± 2.0[a]

Cell cycle distributions are statistically different (Two-way ANOVA) both due to selenocompound treatment and the TR1 attenuation (n=3 for all groups). '[a]' indicates $p<0.01$ versus matched DMSO vehicle control; '[b]' indicates $p<0.01$ versus matched miNeg treatment as measured by the Holm-Sidak posthoc test.

and the selenazolidines increased cells in G_1 phase, but to a lesser extent in miR-TR1 cells than miNeg cells.

To determine if the selenazolidine-mediated cytotoxicity observed in the A549 cells translated to other non-small cell lung cancer lines, we treated H1666 cells (which do not have a KEAP1 mutation) with the cytotoxic agents selenocystine and ChSCA. H1666 cells expressed considerably less TR1 than A549 cells (Figure 4.4A), and displayed similar sensitivity to the cytotoxicity of these agents (Figure 4.4B). Selenocystine, which displayed a more modest sensitization in the A549 cells with attenuated TR1, displayed comparable cytotoxicity in these cells, but ChSCA-dependent cytotoxicity was more similar to the miR-TR1 A549 cells.

Effects of TR1 Knockdown and Glutathione Depletion on Cell Viability

As oxidative stress is a major inducer of cell death, we investigated whether TR1 and GSH depletion together decrease cell viability independent of any external insult. To determine the effect of simultaneously depleting these two antioxidant systems, miR-TR1 cells were treated with BSO. BSO decreases GSH synthesis via inhibition of gamma-glutamylcysteine synthetase, thereby decreasing the cellular GSH concentration. Treatment of A549 miR-TR1 cells with BSO resulted in decreased cell viability in a time-dependent manner, as a difference in cell viability was observed after 72 hours of BSO treatment (Figure 4.5). At this time point, BSO decreased miR-TR1 cell viability by 75% but did not affect the viability of miNeg cells.

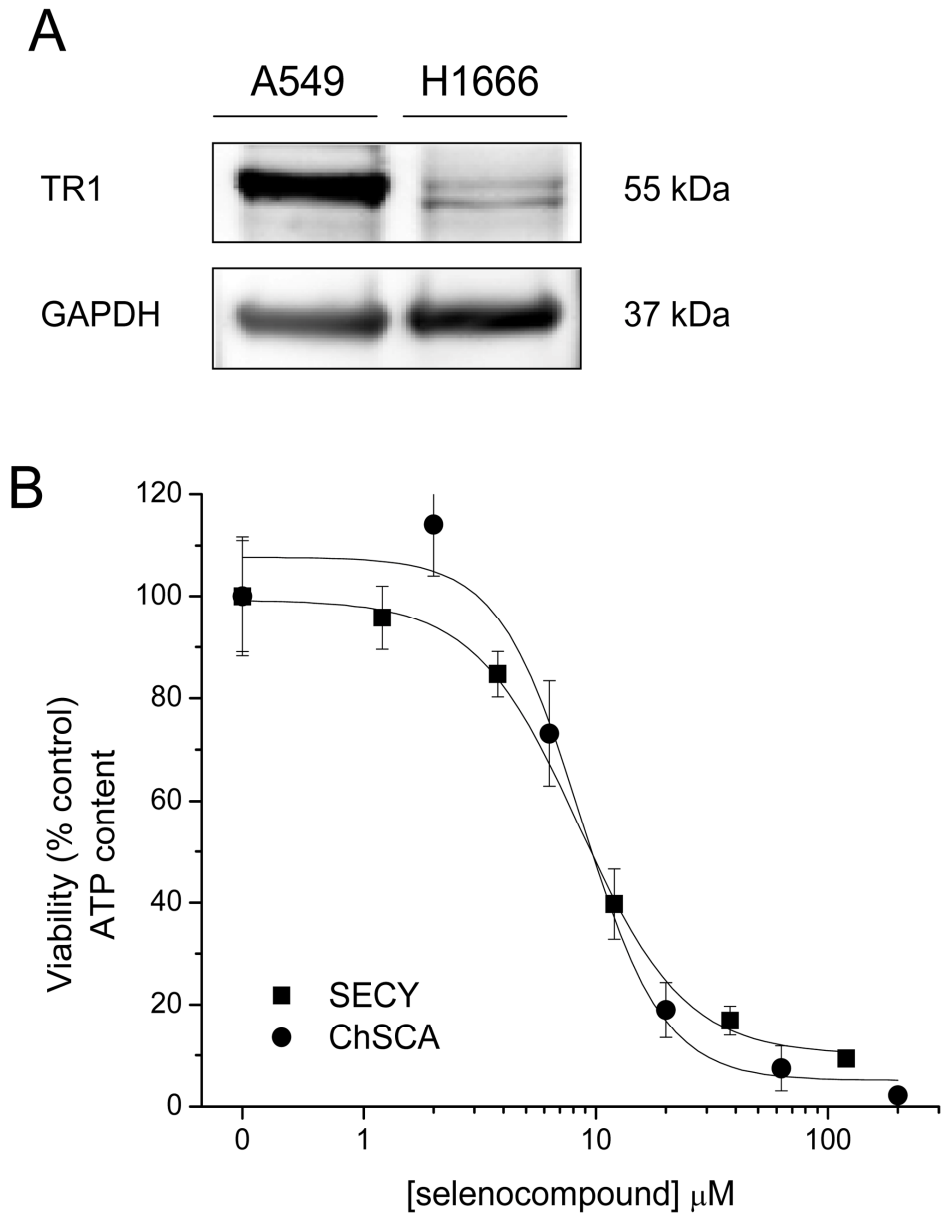


Figure 4.4. TR1 expression and selenocompound cytotoxicity in H1666 human NSCLC cells. (A) Determination of TR1 protein expression by Western blotting. 10 μg protein was loaded for each sample. H1666 cells have ~85% less TR1 protein expression than A549 cells as determined by densitometry. **(B)** H1666 cell viability following 48 hr selenocompound treatments. Concentrations ranged from 0-200 μM (ChSCA) or 0-120 μM (SECY). Viability was assessed by ATP content. Values are expressed as % relative to vehicle controls, which were set to 100%, and n=6 for all data points. Both SECY and ChSCA have similar cytotoxicity (SECY IC_{50}: 8.5 ± 0.6; ChSCA IC_{50}: 8.6 ± 1.7 μM).

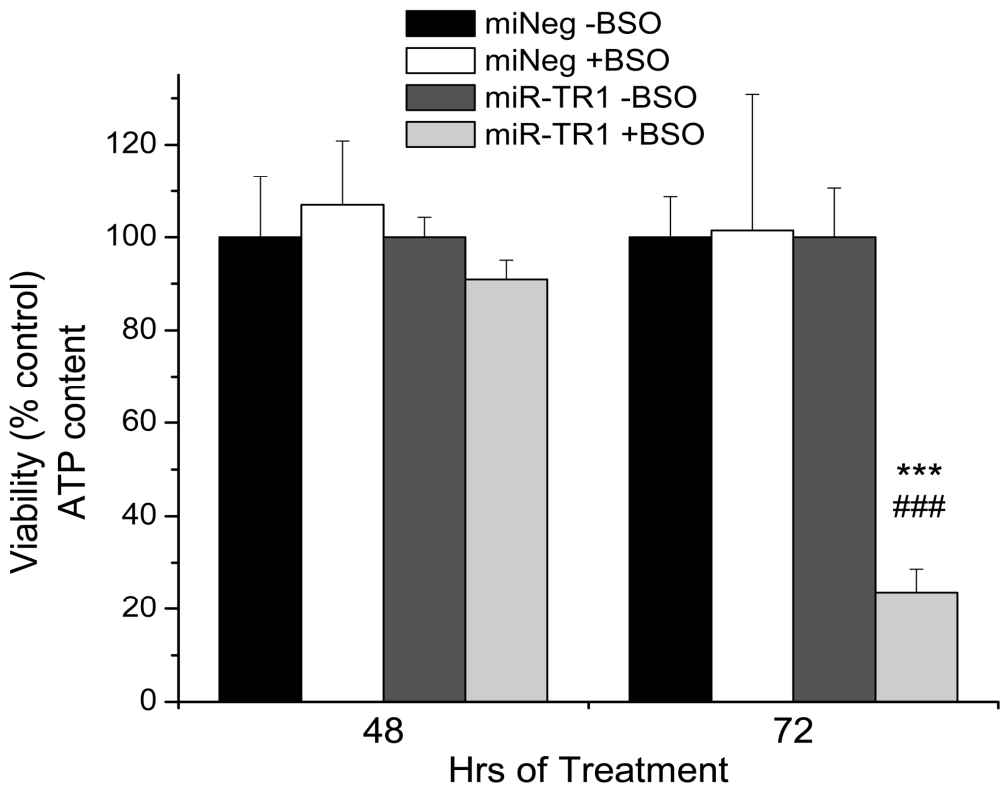


Figure 4.5. Effects of TR1 and GSH attenuation on A549 cell viability. miNeg and miR-TR1 cells were treated with 20 μM BSO or PBS vehicle for 48 or 72 hours. Cell viability was determined by measuring cellular ATP content. BSO-treated viability values are expressed as % matched vehicle controls. n=8 for all groups. '***' indicates $p<0.001$ versus -BSO (control); '$^{###}$' indicates $p<0.001$ versus 48 hr miR-TR1 +BSO.

Selenocompounds Alter Redox Balance in TR1 Knockdown Cells

As the greatest differences in cell viability between miNeg and miR-TR1 cells at 48 hours were observed at low micromolar concentrations of BSCA, ChSCA, and selenocystine, we chose 5 μM treatments of these compounds to further investigate their mechanism of cell death specific to miR-TR1 cells. ROS were increased 2-fold and 4-fold by the selenazolidines and selenocystine, respectively, in the miR-TR1 cells (Figure 4.6A). No increase in ROS was detected with these compounds in miNeg cells, indicating that TR1 may function in A549 cells to protect against ROS generation observed with these compounds. No change in ROS levels was observed at 48 hours with 20 μM selenomethionine or 1 μM MSA. To further evaluate the role of ROS, cells were pretreated with NAC, a thiol antioxidant, or tiron, a nonthiol antioxidant, prior to selenocompound treatment. No attenuation of selenocystine or selenazolidine cytotoxicity was observed with either NAC or tiron (data not shown).

Since GSH depletion decreased A549 cell viability in combination with TR1 knockdown, we measured total intracellular GSH following 48 hr drug treatments to determine if decreased GSH was involved in the increased sensitivity of the miR-TR1 cells. No alteration in GSH concentration was observed with TR1 knockdown alone (Figure 4.6B). The greatest depletion of total GSH was observed with BSCA, ChSCA, and selenocystine, as these compounds decreased total intracellular GSH by 90% independent of TR1 status. CDDP also depleted GSH in a TR1 independent manner, but to a lesser extent than that elicited by selenocystine or the selenazolidines (~40%). Taken together, these data indicate that selenazolidines and selenocystine can alter the cellular redox balance by generating ROS and depleting total intracellular GSH.

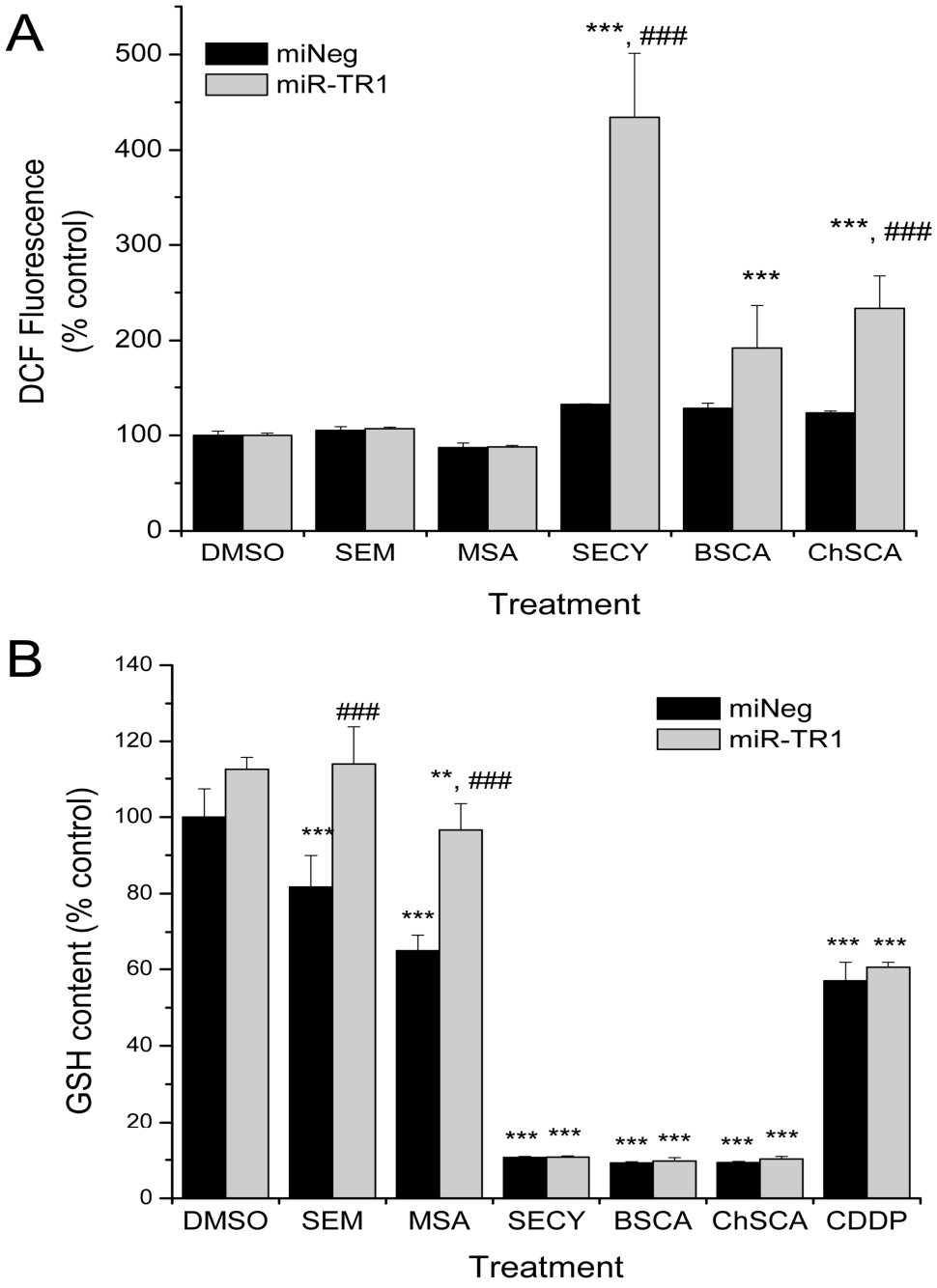


Figure 4.6. Assessment of cellular redox status parameters. Treatments were for 48 hours at the following concentrations: SEM, 20 μM; MSA, 1 μM; SECY, BSCA, and ChSCA, 5 μM; CDDP, 20 μM. **(A)** DCF fluorescence concentration. **(B)** Measurement of total intracellular GSH. GSH concentration values were normalized to cell viability fluorescence values and are expressed as % miNeg DMSO control. n=3 for all DCF assay treatment groups; n=4 for GSH assay treatment groups. '**' indicates $p<0.01$ versus matched DMSO vehicle control; '***' indicates $p<0.001$ versus matched DMSO vehicle control; '$^{###}$' indicates $p<0.001$ versus matched miNeg treatment.

Selenocompound-induced Trx Oxidation

The alterations in ROS and GSH indicative of altered redox balance led us to look at Trx redox status since it can be modulated by oxidative stress and is known to be involved in apoptotic signaling. Trx contains five cysteine (Cys) residues and therefore can exist in six possible oxidation states. In DMSO-treated miNeg and miR-TR1 cells, TR1 was primarily in the reduced state (Figure 4.7; Table 4.2). This finding that TR1 knockdown alone does not alter the Trx oxidation status of A549 cells is in agreement with reports for other transformed cell lines [28, 30]. BSCA, ChSCA, and selenocystine increased the expression of oxidized forms of Trx, primarily the two residue oxidation state, in a TR1-independent manner. No appreciable expression of Trx in the three highest oxidation states was detected with any of the treatments.

To assess the involvement of Trx oxidation in the cell death mechanism of these selenocompounds, we investigated the ASK1 pathway. ASK1 is an apoptotic signaling protein that is regulated by the oxidation status of Trx [31, 32]. ASK1 associates with Trx^{red}, which prevents its phosphorylation and subsequent activation. When Trx is oxidized, ASK1 dissociates and can be phosphorylated, activating apoptotic pathways involving JNK and p38 MAP kinase. As BSCA, ChSCA, and selenocystine increased Trx^{ox} levels, we sought to determine if these compounds were inducing cell death through the ASK1 pathway. Prior to selenocompound treatment, cells were transfected with HA-tagged ASK1 wt or kinase mutant (KM) constructs [31] or treated with 55 nM JNK inhibitor VIII or 600 nM p38 MAP kinase inhibitor SB203580. Despite the increased Trx oxidation observed in both cell lines with the selenazolidines and selenocystine, there was no effect of the ASK1 KM, JNK inhibition, or p38 inhibition on selenocompound-

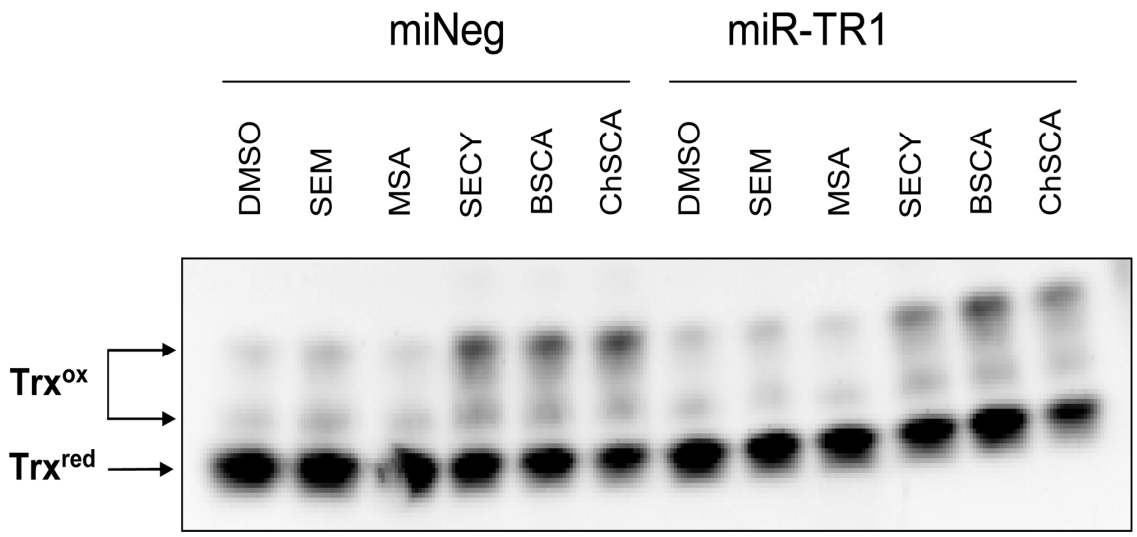


Figure 4.7. Selenocompounds induce expression of Trx in an oxidized state. Iodoacetic acid/iodoacetamine-labeled samples were collected following 48 hr treatments at the following concentrations: SEM, 20 μM; MSA, 1 μM; SECY, BSCA, and ChSCA, 5 μM. Equal amounts ofprotein were analyzed by urea-PAGE and transferred to PVDF membranes that were probed with 1:200 anti-Trx primary antibody. Bands correspond to reduced Trx (bottom band) or Trx in various oxidation states (upper two bands). SECY, BSCA, and ChSCA increased the expression of Trx in an oxidized state in both A549 miNeg (left six lanes) and miR-TR1 (right six lanes) cells. The blot shown is representative of at least two independent experiments.

Table 4.2: Summary of Trx oxidation states.

Cell type	Redox state	Treatment					
		DMSO	SEM	MSA	SECY	BSCA	ChSCA
miNeg							
	4 (ox)	4.4 ± 1.6	6.3 ± 4.5	4.5 ± 0.5	25 ± 2.7	26 ± 1	34 ± 0.1
	5	5.6 ± 1.8	8.1 ± 3.3	5.5 ± 2.2	11 ± 5	9.6 ± 1.8	9.5 ± 2.9
	6 (red)	90 ± 3.4	85 ± 7.8	90 ± 2.6	64 ± 8.4	64 ± 2.9	56 ± 3.4
miR-TR1							
	4 (ox)	7.4 ± 0.2	6.3 ± 1.7	4.9 ± 0.5	22 ± 4.9	28 ± 4	33 ± 8.7
	5	5.9 ± 2.5	4.2 ± 1.1	4.4 ± 1	9.9 ± 1.2	7.3 ± 2.9	9.2 ± 0.8
	6 (red)	87 ± 2.3	89 ± 2.8	91 ± 0.5	68 ± 3.4	64 ± 0.8	57 ± 7.8

Relative band intensities from the Trx oxidation state immunoblot analysis (example in Figure 5) were quantified using densitometry. Three of the six possible oxidation states (the reduced state = 6 (red), one cysteine oxidized = 5, or two cysteines oxidized = 4 (ox)) were detected at relative band intensities greater than 1%. The data are presented as the mean percent relative net band intensities ± standard deviations. n=3 for all samples.

induced cell death (data not shown). These findings suggest that although the selenazolidines and selenocystine alter Trx redox status, they do not induce cell death through the ASK1 pathway.

Mitochondrial Dysfunction in the Mechanism of Selenocompound-Induced Cell Death

To verify that the selenocompounds were inducing cell death and not simply depleting cellular ATP, mitochondrial membrane potential was assessed using the JC-1 assay. TR1 knockdown alone did not affect the mitochondrial membrane potential as there was no difference in the polarized and depolarized populations of miNeg and miR-TR1 DMSO-treated cells (Figure 4.8). TR1 knockdown sensitized the A549 cells to mitochondrial depolarization induced by the selenazolidines, as an increase in the depolarized mitochondrial population was only observed with these compounds in the miR-TR1 cells. Knockdown also further sensitized cells to depolarization by selenocystine. Although 1 μM MSA did not induce cell death as assessed by ATP content, MSA-treated miR-TR1 cells had a greater depolarized mitochondria population than MSA-treated miNeg cells, but this population was not statistically different from DMSO-treated miR-TR1 cells. Consistent with the lack of effects on cytotoxicity and redox parameters in either cell line, selenomethionine did not alter the mitochondrial membrane potential. These finding suggest that the selenocompounds are indeed inducing cell death, as mitochondrial membrane depolarization is a hallmark of the cell death process.

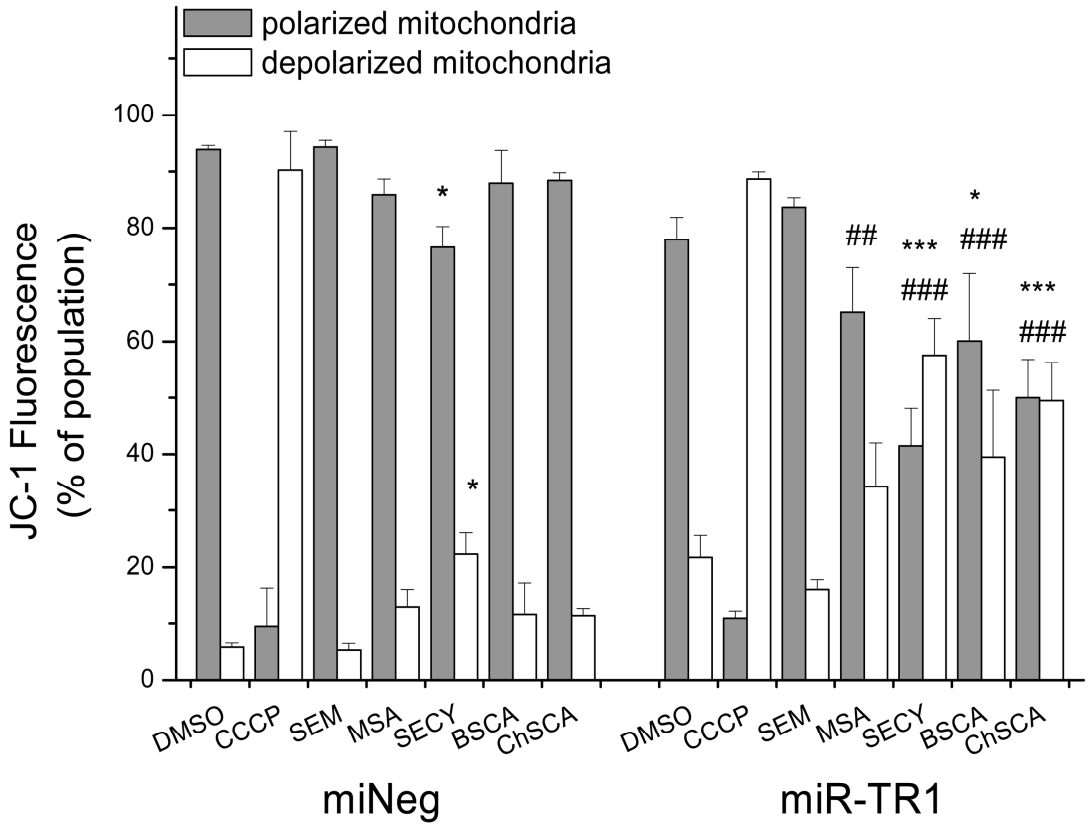


Figure 4.8. TR1 knockdown increases selenocompound-mediated mitochondrial membrane depolarization. Determination of mitochondrial membrane potential by JC-1 after 48 hr treatment. Compounds were used at the following concentrations: SEM, 20 μM; MSA, 1 μM; SECY, BSCA, and ChSCA, 5 μM. Data are expressed as % total population, with the 525 nm fluorescence population (JC-1 green) represented as depolarized mitochondria and the 575 nm fluorescence population (JC-1 red) represented as polarized mitochondria. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP; 25 μM), a known mitochondrial membrane disrupter, served as positive control. '*' indicates $p<0.05$ versus matched DMSO vehicle control; '***' indicates $p<0.001$ versus matched DMSO vehicle control; '$^{\#\#}$' indicates $p<0.01$ versus matched miNeg treatment; '$^{\#\#\#}$' indicates $p<0.001$ versus matched miNeg treatment.

Caspase-Independent Mechanism of Cell Death

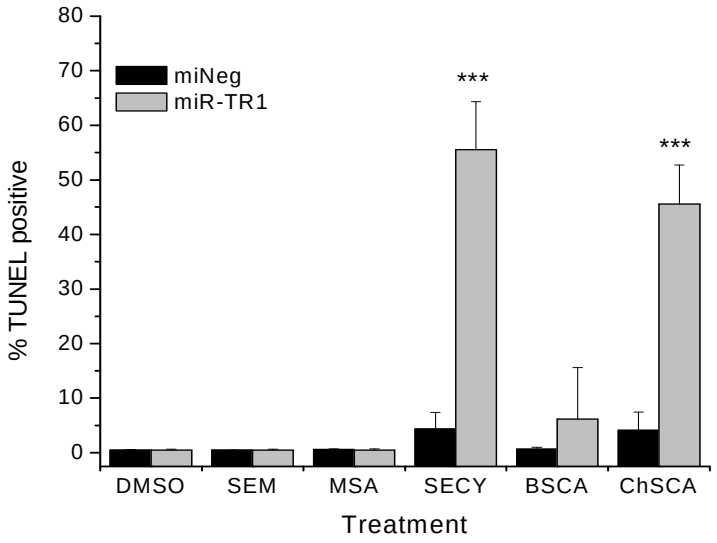
Involving DNA Fragmentation

We investigated the ability of selenocompounds to induce DNA fragmentation using the TUNEL assay to further elucidate the mechanism of cell death. ChSCA and selenocystine induced DNA strand breaks, but only with TR1 knockdown (Figure 4.9A). Surprisingly, BSCA did not produce significant DNA fragmentation with 48 hr treatment despite its ability to decrease cellular ATP content and depolarize the mitochondrial membrane. We sought to further characterize selenocystine and the selenazolidines by determining if they induce cell death in the miR-TR1 cells through a caspase-dependent pathway. Although these compounds elicit mitochondrial membrane depolarization and DNA strand breaks, no increase in caspase 3/7 activity was observed (Figure 4.9B) and selenocompound cytotoxicity was not attenuated when a broad spectrum caspase inhibitor (Z-Asp-CH_2-DBM) was used (data not shown).

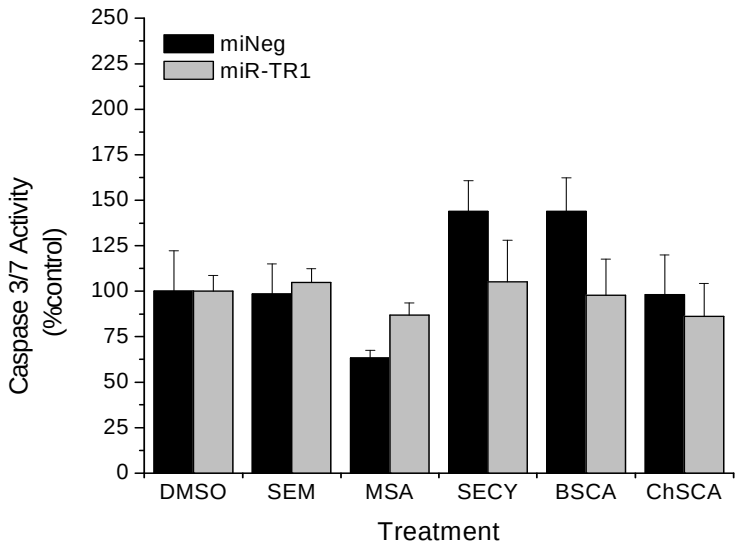
Since there was no indication of caspase activation, we looked for evidence of a caspase-independent mechanism of cell death involving apoptosis inducing factor (AIF). AIF is a mitochondrial protein that translocates to the nucleus upon mitochondrial outer membrane permeabilization, where it induces DNA fragmentation and ultimately apoptosis independent of caspases [33]. Decreased AIF expression in the mitochondrial fraction was observed in miR-TR1 cells with 48 hr selenazolidine and selenocystine treatments as determined by Western blotting (Figure 4.9C). This decreased AIF expression is indicative of AIF translocating from the mitochondria to the nucleus. Taken together, these data suggest that caspase-independent AIF activation is involved in the

Figure 4.9. SECY and the selenazolidines induce cell death through a caspase-independent mechanism. For all data shown, cells were treated at the following concentrations for 48 hours: SEM, 20 μM; MSA, 1 μM; SECY, BSCA, and ChSCA, 5 μM. (**A)** Measure of DNA strand breaks by the TUNEL assay. 10,000 events were measured for each sample. Data shown are for BrdU positive, PI negative cell populations and are presented as % total population. '***' indicates $p<0.001$ versus matched DMSO vehicle control. All samples were analyzed at least in duplicate. **(B)** Caspase 3/7 activity. Data are presented as expressed as % matched DMSO vehicle control. No significance difference between treatment groups was found. **(C)** AIF protein expression in mitochondrial fractions. 1 μg mitochondrial protein was loaded for each sample. GAPDH was probed as a protein loading control. α-tubulin protein was not detected (data not shown), indicating that the mitochondrial fractions were free of cytosolic contamination. The blot shown is representative of two individual experiments.

A


% TUNEL positive
80
70
60
50
40
30
20
10
0
miNeg
miR-TR1
***
***
DMSO
SEM
MSA
SECY
BSCA
ChSCA
Treatment

B


Caspase 3/7 Activity
(%control)
250
225
200
175
150
125
100
75
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miNeg
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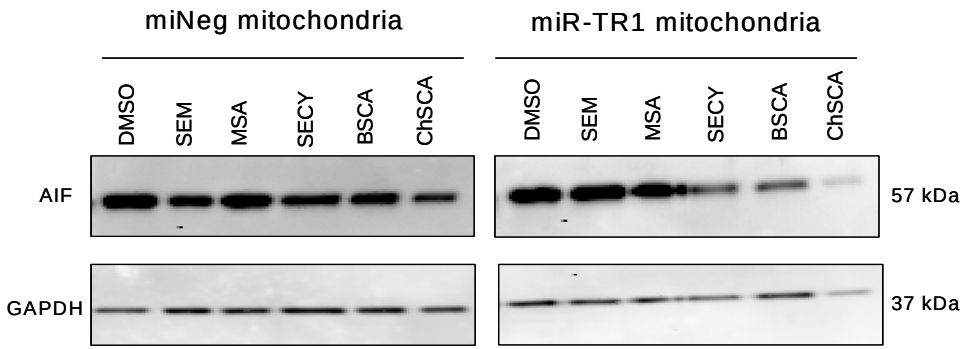
SECY
BSCA
ChSCA
Treatment

C


miNeg mitochondria
miR-TR1 mitochondria
DMSO
SEM
MSA
SECY
BSCA
ChSCA
AIF
57 kDa
GAPDH
37 kDa

cell death mechanism of the selenazolidines and selenocystine at low micromolar concentrations in A549 miR-TR1 cells.

Discussion

Lung cancer has the highest mortality rate of all cancer types, with a 5-year survival rate of only 16% [34]. One reason for this poor survival rate is the low success rate of current treatments. We utilized the A549 human NSCLC cell line as a model of lung carcinoma to further determine the effects of TR1 expression on drug sensitivity. This cell line was chosen because A549 cells have one of the highest overexpression levels of TR1 compared to human bronchial epithelial cells [4]. Additionally, the negative regulator of Nrf2, Keap1, is mutated in this cell line, resulting in constitutive Nrf2 activation [24]. Nrf2 is a transcription factor that regulates the expression of a battery of antioxidant defense genes, including TR1. Thus, A549 cells represent a lung carcinoma with high expression of the Trx system and aberrant Nrf2. Dysfunction of these antioxidant systems has been shown to be a factor in resistance to several anticancer drugs [8, 10, 35, 36]. Altered expression levels of Nrf2 and Keap1 are common occurrences in NSCLC tumors and are associated with worse overall patient survival [24, 37]. Of the lung tumors studied, 26% had nuclear Nrf2 expression, 56% had undetectable or low KEAP1 expression, and 41% had a KEAP1 mutation. An additional study of NSCLC tumors found that almost half of the tumors studied had strong TR expression [4]. These Nrf2, Keap1, and TR1 expression patterns observed in patient tumor samples are similar to those of A549 cells, making this cell line an applicable model for many NSCLCs. In this study, we performed knockdown of a single Nrf2-regulated antioxidant

gene, TR1. There was no observable difference in A549 viability with TR1 knockdown alone in our study or a previous report [38]. We also observed a lack of effects on redox parameters and mitochondrial membrane integrity with TR1 knockdown alone. One explanation for this is the unregulated antioxidant response and increased reductive capacity of the A549 cell line. We extended the observation from the KEAP1 mutant phenotype under study with a comparison of the cytotoxic activity of selenocystine and ChSCA in H1666 cells. The selenazolidine, ChSCA, demonstrated cytotoxicity comparable to the miR-TR1 A549 cells.

Studies have demonstrated decreased anticancer drug resistance with Nrf2 knockdown and the consequential global effects on Nrf2-regulated gene transcription. We observed that knockdown of only one Nrf2-regulated gene, TR1, was able to enhance selenocompound cytotoxicity. The data presented here demonstrate that selenocompounds exhibit differential cytotoxic effects in A549 cells with decreased TR1. TR1 knockdown only increased selenazolidine and selenocystine cytotoxicity as determined by cellular ATP content. Our clonogenic survival assays suggest that the long-term treatment with these compounds may preclude the advantage of TR1 attenuation; however, additional experiments, perhaps including xenograft studies, may be more applicable to determining if targeting TR1 while treating with redox active agents provides therapeutic advantage *in vivo*. TR1 attenuation did not alter the cytotoxicity of MSA, selenomethionine, or *p*-XSC (data not shown for *p*-XSC). Increased sensitivity to MSA has been observed in the RKO colon cancer cell line with TR1 knockdown via an autophagic mechanism [12], indicating that while MSA can have increased cytotoxic properties with TR1 knockdown, these effects are tissue specific. We

also investigated CDDP since the Trx system has been linked to its resistance. CDDP interacts with TR1 at the selenocystine residue, resulting in irreversible inhibition of the enzyme [39]. TR1 knockdown did not affect CDDP cytotoxicity in this study, indicating that other factors may be responsible for CDDP resistance *in vitro*, such as the glutaredoxin system that can be inhibited by the GSH adduct of CDDP. However, it was recently shown that CDDP with the organoselenium TR inhibitor ethaselen reduced tumor size in A549-grafted nude mice to a greater extent than CDDP alone [8], demonstrating that targeting TR1 in combination with redox active anticancer drugs is an effective strategy to reduce tumor growth *in vivo*.

Interestingly, the selenazolidines and selenocystine were the only compounds whose cytotoxicity was affected by TR1 expression. These compounds also increased ROS in a TR1 dependent manner, indicating a protective role for TR1 in A549 cells. TR1 has been shown to protect other cancer cell lines from oxidative species generated by hydrogen peroxide, radiation, and CCDP. It is also possible that knockdown of TR1 lowers the concentration at which selenocompounds function as pro-oxidants rather than antioxidants. The selenazolidines and selenocystine decreased intracellular GSH levels to the greatest extent (~90%). Given that these compounds decreased total GSH independent of TR1 status, it is not surprising that they also affected the Trx oxidation state regardless of TR1 expression. However, GSH depletion and Trx oxidation are unlikely to be solely responsible for the cell death mechanism, as these compounds altered these parameters to an equal extent in the miNeg cells but did not alter miNeg cell viability. Pretreatment with the antioxidants NAC and tiron did not decrease the sensitivity of A549 cells to the selenazolidines and selenocystine. Therefore, either the

pretreatment regimen was ineffective or the ROS generation was a consequence rather than initiator of cell death. Cell death was also observed with depletion of both the TR1 and GSH systems in the absence of an external oxidative or electrophilic insult, indicating the need for depletion of both these antioxidant systems to induce cell killing in the basally reduced A549 cell line. In one study, the depletion of TR1 and GSH together for 48 hours did not result in A549 cell death [38]; we show here that it is necessary to maintain simultaneous depletion of these antioxidant systems out to 72 hours to elicit cell death. This need for sustained antioxidant depletion further demonstrates the high reserve antioxidant capacity of the A549 cell line and presents a potential treatment strategy for these types of tumors.

As one selenocystine molecule contains two selenium atoms, the selenium content of one mole of selenocystine is twice that of compounds containing one selenium atom, such as the selenazolidines. To ensure that the observed cytotoxic and redox effects of selenocystine were not merely a result of a higher selenium concentration from this compound, ROS generation was evaluated for 2.5 μM selenocystine. This concentration provided an equivalent molar amount of selenium as 5 μM BSCA or ChSCA. No increase in ROS was detected with 2.5 μM selenocystine in miR-TR1 cells (data not shown), indicating that the selenium concentration itself is not solely responsible for the observed redox effects. Of course, this assessment of selenium content and selenium-mediated cytotoxicity cannot account for potential differences in absorption, metabolism and elimination that may exist among the selenocompounds *in vivo*. These parameters have yet to be assessed in an *in vivo* model system.

Mitochondrial dysfunction and AIF-induced cell death, such as that induced by the selenocompounds in this study, has been shown to be an effective method of killing chemoresistant NSCLC cell lines [40, 41]. Consistent with our results, evidence for selenocompounds inducing a caspase-independent mechanism of cell death in transformed cells through mitochondrial pathways has been demonstrated, including AIF-mediated mechanisms for selenocystine and selenite [42-45]. In this work, we observed that selenocompounds in combination with TR1 knockdown can induce mitochondrial dysfunction at lower concentrations than those used in our previous study, even as the mitochondrial isoform remains intact. Since caspase-dependent mechanisms of cell death are not effective at killing resistant NSCLC cells, the caspase-independent mechanism indicated by our results presents an intriguing ability of the selenazolidines to induce cell death with decreased TR1 expression.

Selenazolidines are organoselenocompounds designed to release selenocysteine either enzymatically or through spontaneous hydrolysis [46]. Once selenocysteine is liberated, it can dimerize with itself to generate selenocystine. These prodrugs lack the chemical instability associated with selenocysteine, as selenocysteine can easily oxidize to diselenide. Selenazolidines exhibit decreased cytoxicity and greater biological availability of Se in comparison to sodium selenite and selenomethionine in cell culture [47, 48] and comparable chemoprevention efficacy to selenocystine [49]. In this study, we utilized two selenazolidines, BSCA and ChSCA, which are thought to release selenocysteine through spontaneous hydrolysis and have demonstrated anticancer activity *in vivo*. Herein, we have demonstrated that the cytotoxic and redox modulatory properties of the selenazolidines relate to TR1 expression and mirror those of selenocystine.

In summary, our data demonstrate that TR1 knockdown increases the cytotoxicity of the selenocompounds BSCA, ChSCA, and selenocystine in A549 cells through a mitochondrial pathway, as summarized in Figure 4.10. Further work to investigate the use of these compounds in combination with thioredoxin reductase inhibitors or current chemotherapies is of interest.

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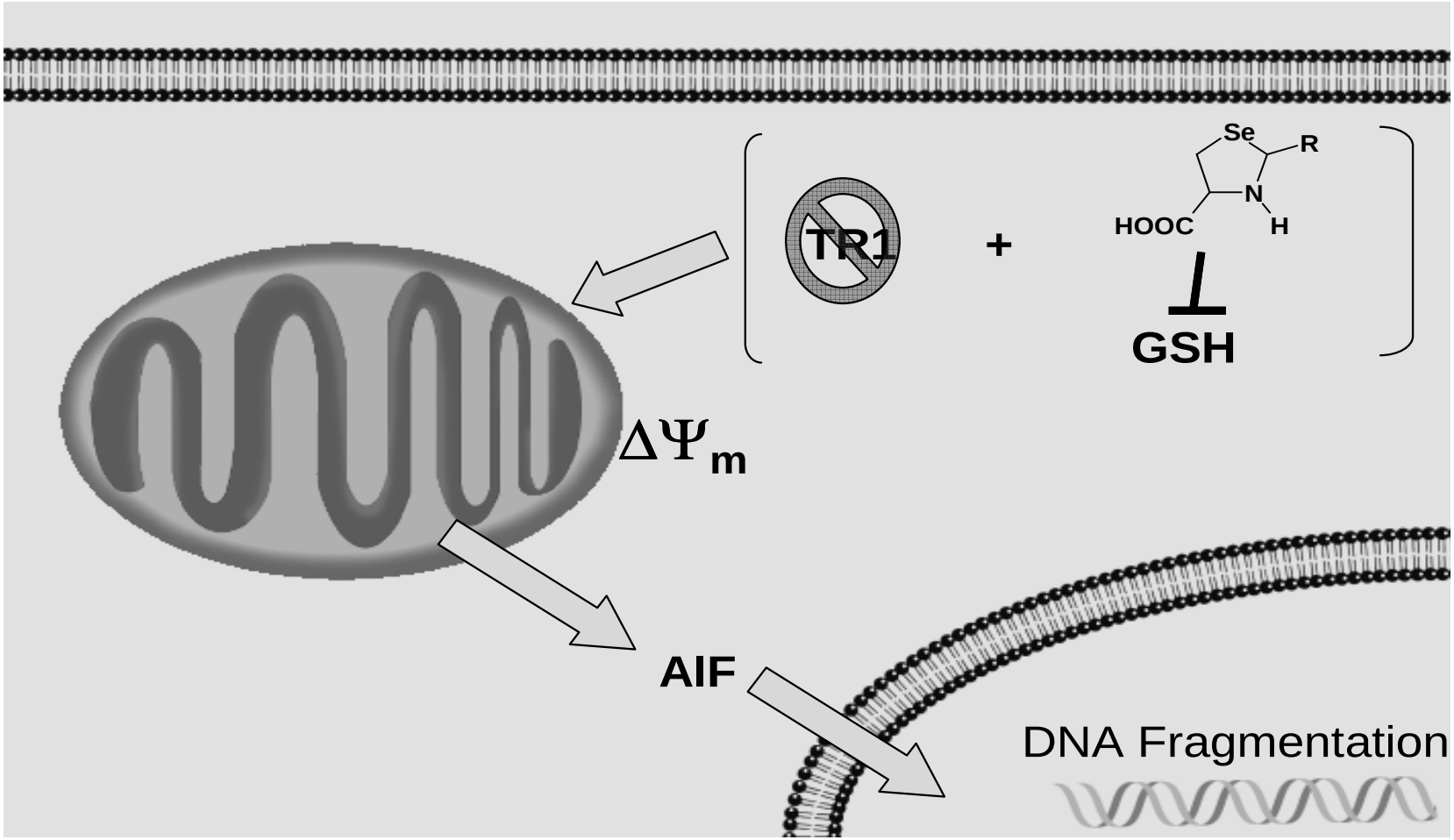

Figure 4.10. Proposed mechanism of cell death by selenocompounds in combination with thioredoxin reductase inhibition in A549 cells. Inhibition of the glutathione system by selenocystine, BSCA, and ChSCA coupled with thioredoxin system inhibition by siRNA leads to mitochondrial dysfunction as indicated by mitochondrial membrane polarization. Apoptosis inducing factor is released from the mitochondria, where it translocates to the nucleus to induce DNA strand breaks and ultimately caspase-independent cell death.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Summary

The overall aim of this work was to evaluate the ability of selenocompounds to modulate the redox status of lung cells and consequently activate pathways associated with cancer prevention or cell death. This dissertation has presented data to support the hypothesis that selenocompounds can induce an oxidative or reductive stress in a compound and cell-line specific manner. Findings from Chapters 3 and 4 demonstrate that selenocystine and ChSCA may have applications both as chemotherapy agents to prevent tumorigenesis by activating the Nrf2/ARE pathway and as anti-cancer agents in combination with TR1 attenuation to induce cell death through oxidative stress. BSCA remains to be evaluated for its ability to activate Nrf2, but was also shown to increase oxidative stress and cell death with TR1 attenuation. MSA produced a reductive stress in A549 cells and a moderate oxidative stress in BEAS-2B cells that could activate the Nrf2 pathway, but its toxicity was not affected by TR1 status. Selenomethionine was unable to modulate redox status and did not activate the Nrf2 pathway in nonmalignant cells or a caspase-independent cell death pathway in malignant cells. These findings will be beneficial in determining which form of selenium and what type of cell line (malignant or nonmalignant) to use in future studies and will also contribute to predicting how a

given selenocompound may affect cellular redox status. This work also indicates novel mechanisms of selenium in cancer prevention and treatment for future study.

Discussion

A potential dual action of selenocystine and ChSCA related to malignancy state is indicated by this work (Figure 5.1). These compounds produced a shift in cellular redox tone to the oxidative side, but with different consequences in the BEAS-2B cells compared to the A549 cells. In nonmalignant BEAS-2B cells, the two compounds initially generated ROS species and depleted intracellular glutathione levels in a rapid manner (2 hours) but had increased levels to above baseline by 24 hours. These compounds decreased intracellular glutathione levels in the malignant A549 miNeg and miR-TR1 cells, but not as rapidly as in the BEAS-2B cells, as the decreases were observed following 48 hour treatment of the A549s. These decreases in glutathione content do not appear to be related to the toxicity of the selenocompounds, as the 5 μM concentrations used in both of the A549 cell lines only decreased viability of the A549 miR-TR1 cells, but depleted glutathione in both the A549 miNeg and miR-TR1 cells. Additionally, selenocystine and ChSCA depleted glutathione to a similar extent at 2 hours in the BEAS-2B cells even though the 1 μM selenocystine concentration was less toxic than the 25 μM ChSCA concentration. Selenocystine and ChSCA also increased ROS levels at 48 hours, but only in the context of TR1 knockdown. It is likely that the generation of ROS at the 48 hour time point is a consequence of cell death, as it is occurring at the same point as the decrease in cellular ATP content and pretreatment with the antioxidants tiron and NAC did not attenuate cell death. At the earlier 24 hour time

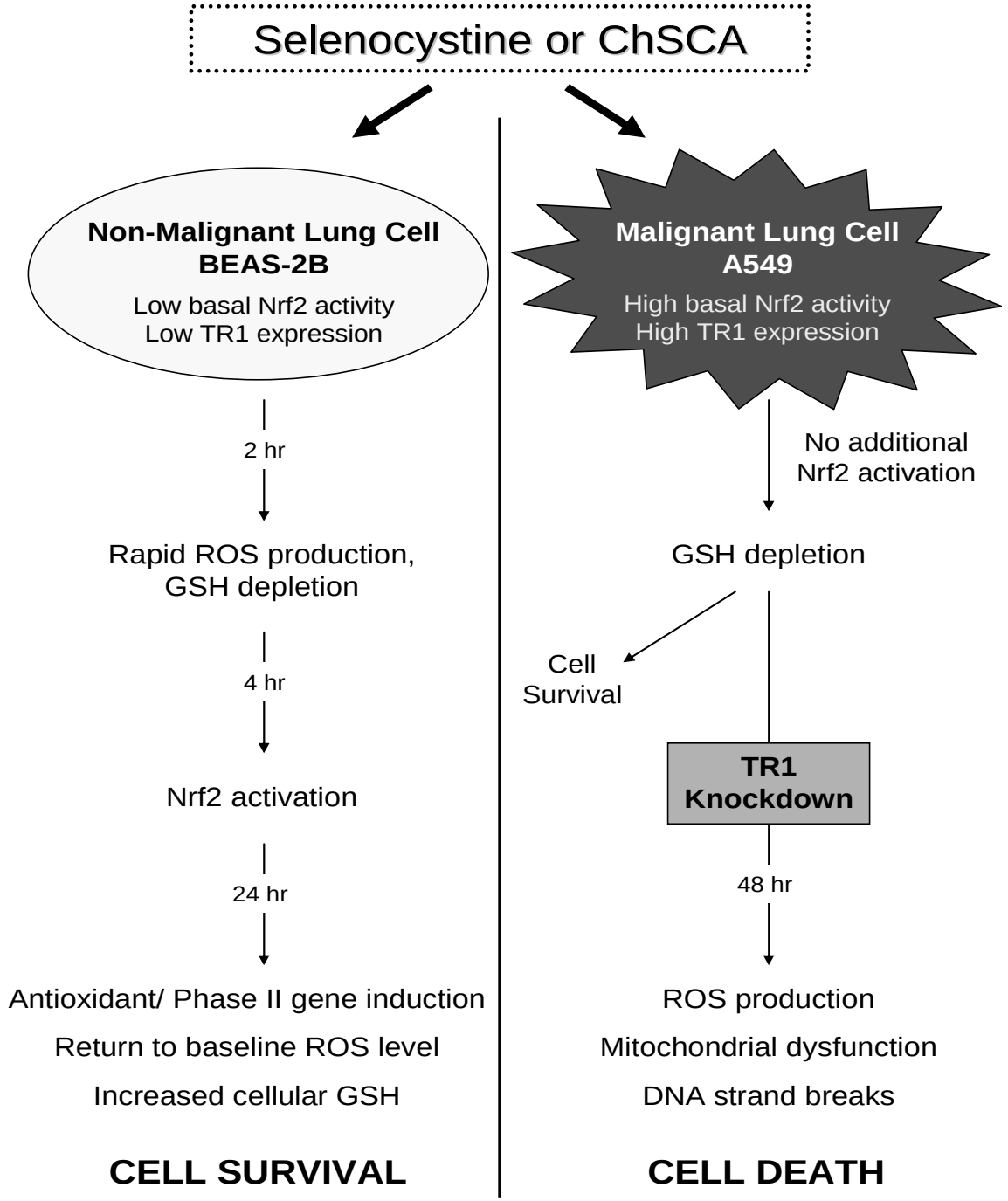

Figure 5.1. Cell fate following treatment with SECY and ChSCA can be malignancy status and basal redox state dependent. In a nonmalignant lung cell with low basal Nrf2 and TR1 expression (BEAS-2B), SECY and ChSCA activate the Nrf2 pathway, thus increasing antioxidant defense to contribute to cell survival. MSA also activates this pathway. In a malignant lung cell with high basal Nrf2 activity and TR1 expression (A549), SECY and ChSCA alone do induce cell death. However, delayed cell death can occur with these compounds, and also BSCA, in the context of TR1 knockdown. Selenomethionine is not implicated in either pathway.

point, selenocystine increased ROS levels in A549 cells, while ChSCA did not. This difference between selenocystine and ChSCA is most likely attributed to the higher toxicity of the 100 μM selenocystine concentration used at the 24 hour time point (~60% viable cells) compared to the 100 μM ChSCA concentration (~100% viable cells). This difference in cell viability with these selenocystine and ChSCA concentrations did not result in differential effects on free thiol levels or mitochondrial membrane potential in the A549 cells at 24 hours, as both measurements were similarly affected by selenocystine and ChSCA.

Although the generation of selenocystine from the selenazolidines has yet to be formally evaluated, the similar actions of ChSCA to selenocystine described in Chapters 2, 3, and 4 indicate that ChSCA may indeed release selenocysteine as it was designed to do. BSCA also demonstrated similar effects as selenocystine in the A549 miNeg and miR-TR1 cell lines. Taken together, these data indicate that the observed effects of these two selenazolidines is likely due to their functioning as selenocysteine prodrugs to release selenocystine that may then form the more stable selenocystine dimer *in vitro*.

The effects of MSA on cellular redox status also varied between the A549 and BEAS-2B cell line even though the 2.5 μM concentration resulted in ~70% viability of both cell lines at 24 hours. In the A549 cells, MSA was shown to produce a reductive stress and increase expression of the ER stress marker BiP/GRP78. This finding is in agreement with studies showing MSA generates ER stress in prostate [1, 2] and colon [3] cancer cells. TR1 knockdown in the A549s did not modulate the toxicity of MSA, in contrast to the oxidative compounds selenocystine, ChSCA, and BSCA. It is possible that TR1 does not regulate ER stress or the unfolded protein response and thus its knockdown

would not further increase cell death from MSA-mediated ER stress. As TR1 is localized to the cytosol, it is unlikely that it has direct actions in the ER. This is supported by the finding that TR1 knockdown in RKO colon cancer cells did not increase ER stress generated by MSA [3]. Another possibility is that reductive stress imparted by MSA does not consume as much glutathione as an oxidative or electrophilic stressor. This less dramatic depletion of glutathione by MSA in the absence of oxidative stress may explain why the toxicity of MSA was not enhanced in A549 cells with TR1 knockdown. In the BEAS-2B cells, MSA did not produce a reductive stress as indicated by altered BiP/GRP78 expression at 24 hours, but rather elicited oxidative stress at the 2 hour time point in these cells. MSA generated ROS and depleted glutathione in BEAS-2B cells at 2 hours, although to a lesser extent than selenocystine or ChSCA at the concentrations utilized. The magnitude of changes in ROS and glutathione with MSA do not appear to be related to cell viability, as the less toxic 1 μM selenocystine (~100% viable cells at 24 hours) depleted glutathione to a greater extent and generated ROS at a similar level compared to 2.5 μM MSA (~70% viable cells at 24 hours). Thus it appears that the redox effects of MSA are dependent on the malignancy status of a cell line.

Of particular note are the findings related to selenomethionine. Selenomethionine is of high interest due to its use as the form of selenium supplementation in SELECT and its high presence in the selenized yeast used for the NPC trial. These trials have shown a modest benefit for selenium in decreasing cancer incidence and mortality [4, 5]. While the results of these two trials may be related in part to subject selenium status, the choice of selenomethionine may also have been a contributing factor [6, 7]. To this end, the present work has shown that selenomethionine does not elicit similar redox changes as

the other selenocompounds investigated. Selenomethionine did not alter cellular ROS or free thiol levels and did not affect mitochondrial integrity, despite being used at a concentration (100 μM) up to 20 times greater than the other compounds investigated. This 100 μM selenomethionine concentration was not associated with decreased viability of the BEAS-2B cells at 24 hours or the A549 cells at either 24 or 48 hours. The most likely explanation for these findings is that selenomethionine does not form the selenide anion for redox cycling unless it first forms methylselenol through a methionase reaction [8], while selenocystine and MSA have been shown to [9], and the selenazolidines are hypothesized to, form the selenide anion. Further, selenomethionine can be incorporated into general cellular proteins and diverted from the hydrogen selenide pool, decreasing the amount of selenium available for selenoprotein biosynthesis, methylselenol formation, and redox cycling. The lack of altered cellular redox status elicited by selenomethionine in this study may explain its lack of efficacy in animal models [10, 11]. These findings for selenomethionine provide further evidence that the form of selenocompound, and not merely the presence of selenium, is a major determinant of observed effects.

The data presented in Chapter 2 indicate a greater sensitivity of the nonmalignant BEAS-2B cells than the malignant A549 cells to the selenocompounds assessed. Previous studies have also investigated the sensitivity of malignant cells to selenium in comparison to that of normal, nonmalignant cells. The results have primarily indicated a greater sensitivity of malignant cells to selenocompounds [12-19], with one study showing nonmalignant cells to be more sensitive [20]. One explanation for the greater toxicity of selenocompounds in malignant cells attempts to relate the toxicity to the glutathione

levels of a given cell line [12]. It is thought that the higher glutathione levels found in malignant cells provide greater amounts of thiols for selenocompounds to redox cycle with, producing more reactive species that would lead to cell death. Another explanation involves higher selenium uptake and accumulation related to a reduced extracellular environment and expression of the x_c^- cystine transporter [21], but this has only been investigated for selenite toxicity. The lower toxicity of the selenocompounds in the malignant A549 cells is in contrast to the idea that cell lines with higher glutathione levels and thus a more reduced cellular redox tone are more sensitive to selenocompounds. In the case of the A549 cells, the higher basal level of glutathione, higher expression of antioxidant defense genes, and a basal reduced state [22] may result in the lower sensitivity of these cells to the selenocompounds as the cells would be better equipped to attenuate oxidative stress generated by the selenocompounds. This is in comparison to the BEAS-2B cells that have lower glutathione levels, lower expression of antioxidant defense genes, and a more oxidative basal redox tone [22], making them more susceptible to selenocompound-induced cell death. In fact, the higher expression of the manganese superoxide dismutase enzyme in normal prostate cells compared to the paired malignant prostate cells was related to the lower toxicity of selenite in the normal cells [13]. Additionally, the data in Chapter 4 indicates that TR1 expression can be a determinant of selenocompound toxicity; the low expression of TR1 in BEAS-2B cells [23] may also contribute to the greater sensitivity of these cells. Expression of genes related to selenium sensitivity, such as selenoprotein 15 [14], may also differ between the two cell lines. Differences in selenium uptake and accumulation may also be responsible

for the differential toxicity profiles of the selenocompounds in the A549 and BEAS-2B cells and have not yet been investigated.

In addition to toxicity, the basal redox tone of a cell may also be an important determining factor in the actions of certain selenocompounds, such as their ability to modulate glutathione and ROS levels, and the time course that such actions follow. It was shown in Chapter 2 that the basal redox status of these two cell lines differs, with the A549 cells being in a more reduced state than the BEAS-2B cells. This can be further extended to the differential basal Nrf2 activity of the A549s and BEAS-2Bs [22], which may be a determining factor in the differential basal redox state of these two cell lines. It is likely that selenocystine and ChSCA undergo redox cycling in both cell lines. In the BEAS-2B cells, Nrf2 activity is low, but is stimulated by the consequences of selenocompound redox cycling, such as ROS generation and glutathione depletion. Nrf2 may also be activated by redox cycling through oxidation of Keap1 reactive cysteines. This would lead to the increased glutathione levels observed in the BEAS-2B cells at 24 hours. In the A549 cells, the basal Nrf2 activity is already high, even prior to selenocompound treatment. When the selenocompounds are introduced and begin to redox cycle, the A549 cells are well prepared to attenuate oxidative stress from their already high Nrf2 activity, antioxidant gene expression, and glutathione levels. By 48 hours, these compounds have depleted glutathione through an unknown mechanism, even though Nrf2 activity should still be high. When TR1 is present, as it is at high levels in A549 cells, this glutathione depletion does not lead to cell death. It is thought that is because TR1 can continue to maintain the cellular redox state for such redox sensitive processes as cell proliferation. However, when TR1 is attenuated and glutathione is

depleted, mitochondrial dysfunction occurs, leading to cell death. This outline serves as one explanation that attempts to integrate the observed findings for selenocystine and ChSCA with the known Nrf2 status of these cell lines.

One limitation of the studies in this dissertation is that it is not known exactly which reactive species are being generated in cell culture by the selenocompounds. H_2DCF-DA was utilized to measure generated ROS, as it is a commonly published protocol used to measure ROS *in vitro*. H_2DCF-DA is a lipophilic compound that is not itself fluorescent. When taken up by cells in culture, the acetate groups on H_2DCF-DA are cleaved, producing the cell membrane-impermeable DCFH. Reactive species then react with DCFH, generating fluorescent DCF. For data in this dissertation, PI fluorescence was also measured in the cytometric assay to assess DCF fluorescence from viable (PI negative) cells. There are conflicting reports as to what oxidants (superoxide radical, H_2O_2) primarily react with DCFH to produce DCF [24]. In addition to ROS, other reactive species may be generated by the selenocompounds that could increase DCF fluorescence. BSCA and ChSCA also generate aldehydes when releasing selenocysteine that could be responsible for their observed downstream effects. However, BSCA and ChSCA behaved similarly to selenocystine, indicating that the actions of these selenazolidines are related more to their release of selenocystine than generation of aldehydes. BSCA and ChSCA were also shown to be less toxic than selenocystine at 24 hours in the BEAS-2B cells and at both 24 and 48 hours in the A549 cells. If these selenazolidines were releasing high concentrations of aldehydes, which are regarded as toxic compounds, they would elicit a more dramatic decrease in cell viability than what was observed. For the purposes of this dissertation, the species generated by the

selenocompounds were not of interest, but rather whether an overall increase in ROS was observed that would be consistent with a shift in cellular redox tone. Intracellular glutathione content was also measured following selenocompound treatments in the A549 and BEAS-2B cells to serve as a further measurement to assess alterations in cellular redox tone.

Mechanism of Chemoprevention

Selenocystine, ChSCA, and MSA were shown to increase antioxidant gene expression through Nrf2 activation, but the mechanism of Nrf2 activation by these compounds is unknown. These selenocompounds were shown to increase ROS levels at 2 hours when used at concentrations that demonstrate less than a 30% loss in BEAS-2B cell viability at 24 hours as shown in Chapter 2. Whether the generation of these ROS is responsible for the disruption of the Keap1/Nrf2 complex and subsequent Nrf2 translocation to the nucleus is currently unknown. While 25 μM ChSCA elicited the greatest fold increase in Nrf2 target gene mRNA expression and produced the greatest loss in cell viability at 24 hours at the selenocompound concentrations utilized, it generated ROS at levels equivalent to those generated by 1 μM selenocystine and 2.5 μM MSA. These findings indicate that perhaps another mechanism in addition to ROS generation is involved in Nrf2 activation by these selenocompounds. Additionally, 5 μM ChSCA also produced a greater increase in TR1, NQO1, and GCL_C mRNA expression in the NHBE cells than 2.5 μM MSA, although both of these selenocompound treatments had similar effects on cell viability (~80% viable cells for both treatment concentrations).

An important step in elucidating the mechanism of Nrf2 activation would be to determine how these selenocompounds modify reactive thiols on Keap1. The sulfhydryl groups of Keap1 cysteines can be modified by oxidation, reduction, or alkylation [25]. Selenocompounds have been shown to interact with protein thiols through direct protein adduct formation [26] or protein thiol oxidation from hydrogen selenide and methylselenol products [26-29]. The Keap 1 cysteines Cys-151, Cys-273 and Cys-288 have been shown to be key targets of Nrf2 activators [30-32], but the Keap1 cysteines modified appear to be compound dependent [25, 33]. One study performed by Kobayashi *et al*. used zebrafish to categorize 11 Nrf2 activators into six classes based on how they activate Nrf2 [25]. This study also indicated that compounds that target specific cysteines may have distinct biological effects [25]. Determining how the selenocompounds activate Nrf2 would allow them to be classed with these other activators that are better characterized in regard to the Nrf2 activation mechanism and may provide further insight into the biological effects these selenocompounds may produce as a result of Nrf2 activation.

The Nrf2 pathway can also be activated by ER stress, leading to the increased expression of such Nrf2 target genes as GCL_C and NQO1 [34, 35]. PERK, a protein kinase involved in the unfolded protein response, is activated following ER stress and phosphorylates eukaryotic initiation factor 2α, resulting in the inhibition of translation initiation [36]. PERK has also been shown to phosphorylate Nrf2 independent of eukaryotic initiation factor 2α phosphorylation, resulting in its dissociation from Keap1 and translocation to the nucleus [34]. Additionally, Nrf2 is implicated in enhancing proteasomal activity [37] and promoting cell survival following ER stress [34, 34, 37].

While it was shown in Chapter 2 that selenocystine, ChSCA, and MSA did not alter BiP/GRP78 protein expression at 24 hours in BEAS-2B cells, it is unknown whether these Nrf2-activating selenocompounds cause ER stress at an earlier time point (e.g., 2 hours) that would result in the translocation of Nrf2 to the nucleus at 4 hours as shown in Chapter 3.

Chapter 3 shows that the selenocompounds can activate the Nrf2/ARE pathway in the lung, but little is known as to whether selenocompounds can activate this pathway in other tissues. The ability of selenocystine and selenazolidines to increase phase II gene expression has been assessed in the liver. No common pattern of increased mRNA or activity by selenocystine and the selenazolidines relating to their observed chemoprevention activity was found when measured 24 hours after a single dose [38] or after a week of daily administration [39]. Differential phase II enzyme induction by the selenocompounds was also observed in Hepa1c1c7 mouse hepatoma cells. ChSCA increased Trxnd1, Nqo1, glutathione S-transferase alpha, mu- and pi classes, and Ugt1a6 mRNA in following 18 hr treatment, and all but the increase in Ugt1a6 were transcriptionally dependent [40]. Selenocystine and selenomethionine moderately increased glutathione S-transferase alpha, and selenocystine also increased Trxnd1, but neither compound increased Nqo1, glutathione S-transferase m or p classes, or Ugt1a6 expression in this system. These previous studies in liver are in slight contrast to the findings for the lung presented in Chapter 3 of this work. However, it is important to note differences in the tissue of interest and animal species. These other works analyzed the liver of male CF-1 mice, while this work is looking at the lung of female A/J mice. Nevertheless, the studies together with the findings in Chapter 3 demonstrate the ability

of selenocystine and selenazolidines to induce phase II enzymes in several tissue types. The induction of these enzymes by selenocystine, selenazolidines, and MSA could be beneficial in other tissues where both selenium and Nrf2 is implicated in cancer prevention, such as the colon [41-43]. It remains to be seen in what other tissues the Nrf2/ARE pathway can be activated in by selenocompounds.

Mechanism of Cell Death with TR Knockdown

The results from Chapter 4 demonstrate that the ability of selenocompounds to deplete glutathione is a major determinant for increased cytotoxcity with TR1 attenuation. The concept of pharmacological inhibition of either the thioredoxin or glutathione systems alone for cancer treatment has been previously investigated. Gold compounds that inhibit thioredoxin reductase, such as auranofin [44, 45], can cause cancer cell death through a mitochondrial pathway [46-48]. The use of BSO to deplete glutathione has been investigated as an adjuvant therapy *in vitro* and in clinical studies [49, 50]. However, inhibition of both antioxidant systems is a novel approach to cancer treatment and represents a synthetic lethality strategy. Synthetic lethality is defined as cell death resulting from the simultaneous inactivation of two genes that do not affect cell viability when independently inactivated [51]. An *in vivo* study investigating the role of TR in tumor growth and the interaction between the thioredoxin and glutathione systems by Mandal *et al*. was published at the same time as Chapter 4 [52]. In keeping with the data presented in Chapter 4, TR knockdown alone did not affect proliferation or the clonogenic and tumorigenic potential of transformed cells [52]. As a compensatory mechanism for attenuated TR expression, the glutathione system was upregulated and

glutathione was required for survival and proliferation of TR deficient cells. The combination of genetic TR knockout and glutathione depletion by BSO decreased xenograft tumor mass, while no effect was seen with attenuation of either system alone. This *in vivo* study further validates the results from Chapter 4 as being an applicable approach to treating tumors in a whole animal system.

The mechanism of selenocompound-induced glutathione depletion, as well as the time course, is unknown. It is also unknown whether glutathione depletion is an initiating factor in cell death or a consequence. One explanation for the findings in Chapter 4 is that knockdown of TR1 places additional pressure on the glutathione system to maintain cellular redox status that becomes oxidative from the selenocompounds and glutathione levels are consequently depleted. This is supported by alterations in glutathione metabolism that occur to compensate for TR1 attenuation [52]. In addition to the evidence for cross-talk between the thioredoxin and glutathione systems in mammalian cells [42], there are also reports in yeast [53], *Drosophila melanogaster* [54] and *Arabidopsis thaliana* [55]. However, this does not explain why glutathione levels decrease but are not returned to baseline levels in the A549 cell line, which should have a high capacity for glutathione biosynthesis from its constitutive Nrf2 activity.

Glutathione export has been implicated in initiating apoptosis for certain compounds such as staurosporine [56]. When glutathione levels were decreased with BSO or glutathione efflux inhibited, staurosporine-induced apoptosis was prevented. The data for the selenocompounds do not support induction of cell death by glutathione efflux, as BSO slightly increased cell death observed with selenocystine, ChSCA, and BSCA (data not shown).

Based on the current data, it is unlikely that the glutathione depletion at 48 hours is due to glutathione-mediated export of the selenocompounds or their metabolites alone. It is expected that if glutathione was depleted through this mechanism, it would occur before 48 hours and cells would regenerate glutathione to replace what was lost in conjugation. Also, the current data do not indicate that reduced glutathione is depleted by providing reducing equivalents to reactive species generated from the selenocompounds, as levels of oxidized glutathione do not increase. Another theory is that selenocystine, BSCA, and ChSCA inhibit the rate-limiting glutathione biosynthesis enzyme glutathione cysteine ligase, perhaps in a manner similar to BSO, which is thought to bind to the enzyme as a glutamate analogue and also block the cysteine binding site [57].

How this attenuation of both the thioredoxin and glutathione systems leads to mitochondrial dysfunction is unknown. Direct modification of protein thiol groups by selenium and a lack of reducing equivalents to return these thiol groups to a reduced state resulting from TR1 and glutathione depletion is one hypothesis. A number of mitochondrial enzymes have critical thiol groups that need to be in the reduced state to properly function [58]. Selenium was shown in cell extracts and isolated mitochondria to decrease mitochondrial membrane potential as a result of protein thiol modification [59, 60]; a similar mechanism may occur in the A549 TR1 knockdown cells treated with selenocystine, BSCA, and ChSCA. Another explanation is that the decrease in antioxidant capacity results in an overwhelming oxidative stress from the mitochondrial generation of ATP and other biofactors that cancer cells need at high levels for their uncontrolled proliferation. The tendency of cancers to overexpress TR1 and have high levels of Nrf2 activity may relate to the high rate of cell proliferation, increased

metabolic demands, and the resultant need to increase antioxidant defense capacity in order to attenuate oxidative metabolites generated during cellular processes related to proliferation [61, 62]. The resultant mitochondrial stress can not be attenuated by the thioredoxin or glutathione systems, which have been depleted, so mitochondrial cell death pathways such as AIF are activated.

As a whole, this dissertation demonstrates that selenocompounds such as selenocystine and ChSCA can have applications in both cancer prevention and cancer therapy through their effects on cellular redox status. This scheme of the dual actions relating to cell survival and cell death is illustrated in Figure 5.1. MSA is also implicated in the Nrf2 activation cell survival pathway, but not the cell death pathway associated with TR1 knockdown. Selenomethionine is not implicated in either pathway. While this scheme is useful for generalizing the actions of two selenocompounds in both nonmalignant and malignant lung cells, several aspects of these pathways are missing, such as how the Nrf2 pathway is activated and how the combination of glutathione and TR1 depletion leads to cell death. Ideas for future studies in regard to these unknown mechanisms are discussed in the next section.

Future Directions

The long term objective of this work is to determine the mechanism action of selenium in chemoprevention and cancer therapy. While the studies described in this work have indicated Nrf2 and mitochondrial dysfunction as mechanisms of prevention and treatment, respectively, these only represent single mechanisms. It is unlikely that only one mechanism is responsible for the effects of selenium and mechanisms may be

organ site specific. Studies of other mechanisms, such as histone deacetylase inhibition [63, 64], and other tissues, such as colon, warrant investigation. In comparison to the other selenocompounds investigated in this work, the selenazolidines are poorly characterized. Studies to further characterize the selenazolidines, such as those related to biodistribution, metabolism and ability to redox cycle, are necessary to further understand their mechanism of action. The selenazolidines have only been investigated in an animal model of lung cancer and should be tested in models of other cancers for which selenium has an indicated benefit, such as prostate and colon. Additionally, the properties of the selenocompounds identified in this work may have applications in additional diseases. One example would be the use of the Nrf2-activating selenocompounds in lung diseases where Nrf2 has a protective role, such as asthma [65] and emphysema [66]. Future work related to either the chemoprevention or anti-cancer mechanisms of selenium is described in the remainder of this chapter.

Chemoprevention Mechanisms of Selenium

Future studies need to be performed on the mechanism of Nrf2 activation by the selenocompounds. It is currently unknown how selenocompounds may modify key cysteine residues on Keap1 that would lead to Nrf2 release and which Keap1 residues the selenocompounds target. This could be determined using mass spectrometry to detect selenocompound-Keap1 adducts or Keap1 cysteine modifications. It is also unknown whether the selenocompounds affect the ubiquitination, and therefore degradation, of Nrf2 or Keap1, mechanisms that have been associated with other Nrf2 activators [67, 68]. By elucidating the mechanism of Nrf2 activation by selenocompounds, Nrf2 activators

with a differential mechanism could be tested for synergy with the selenocompounds to further enhance Nrf2 activation and downstream gene induction.

Selenocompounds may also have other mechanisms to activate Nrf2 in addition to Keap1 modification that could be investigated. Glycogen synthase kinase 3 beta inhibition can lead to nuclear Nrf2 accumulation [69]. MSA has been shown to inhibit glycogen synthase kinase 3 beta [70], but it remains to be seen whether MSA activates Nrf2 by inhibiting this pathway. It was shown in Chapter 2 that ROS levels are increased by selenocompounds prior to Nrf2 translocation to the nucleus, but it is unknown whether this generation of ROS is necessary for Nrf2 activation by the selenocompounds. This could be tested by pretreating cells with antioxidants such as NAC or a superoxide dismutase mimetic to attenuate selenocompound-induced ROS generation and then evaluating nuclear Nrf2 expression and downstream ARE gene expression.

Additional experiments in animal models would further elucidate what effects selenocompounds have on the lung. MSA still needs to be evaluated for chemoprevention in an animal model of lung cancer, as no such study has been published to date. MSA, selenocystine, and ChSCA were shown in Chapter 3 to increase glutathione levels in cell culture. Whether these compounds also increase lung glutathione levels at the organ site, similarly to what has been observed for *p*-XSC [71], is currently unknown. The ability of these compounds to increase lung glutathione levels in the mouse would demonstrate whether this effect observed in cell culture translates to an animal model. Another important aspect of the *p*-XSC mechanism is thought to be its ability to decrease DNA adducts from NNK administration [72]. It is currently unknown whether the other selenocompounds investigated in this work have similar effects on NNK-induced DNA

methylation and warrants investigation. The biodistribution of selenium to the lung has also been related to the efficacy of other selenocompounds in decreasing lung tumor formation [71, 72] and should be assessed in animals administered selenocompounds. Evidence for other cancer types that may benefit from certain selenocompounds would also be generated by determining if organ sites in addition to the lung have increased selenium levels, such as the colon or mammary glands. The inclusion of selenazolidines that were ineffective at decreasing the number of NNK-induced lung tumors in such studies assessing lung glutathione, DNA adduct, and selenium levels would also prove useful in determining whether these actions are important for chemopreventive benefit. Finally, the ability of selenocompounds to decrease lung tumor incidence in an Nrf2 knockout mouse would determine whether Nrf2 is required for the chemopreventive benefit of selenocompounds, an important piece of evidence that is currently lacking.

Selenocompounds in Cancer Treatment

How selenocystine, ChSCA, and BSCA deplete glutathione to levels 20% of baseline is not understood. The current data are only for one time point, 48 hours, so it is unknown how early after treatment glutathione levels begin to decrease. Further experiments establishing a time course of glutathione depletion are needed to better understand how the depletion occurs. Glutathione can be used to detoxify compounds by forming conjugates through reactions with glutathione S-transferases or through reduction reactions, including those with glutathione peroxidases. It is unknown whether glutathione is depleted through such detoxification pathways. This could be determined by measuring glutathione conjugates of selenocompounds and their metabolites or

inhibiting glutathione S-transferases and then measuring glutathione levels. A second possibility to be investigated is the inhibition of the rate-limiting glutathione synthesis enzyme glutamate cysteine ligase by these selenocompounds or their metabolites.

The way by which selenocompounds cause mitochondrial dysfunction in the A549 cells is unknown. The ability of these compounds to modify mitochondrial protein thiols or generate mitochondrial superoxide and mitochondrial DNA mutations should be assessed. Whether the selenocompounds in combination with TR1 knockdown alter cellular metabolism should also be determined, as the observed effects on the mitochondria may result in changes related to metabolism. Cellular aerobic respiration and glycolysis could be measured using an extracellular flux analyzer, such as the one from Seahorse Bioscience Inc. A second strategy would be to utilize phenotype microarrays from Biolog to determine if the carbon metabolism and energy pathways required for cell survival are altered. Further work on cellular metabolism could be performed using mass spectrometry and HPLC to monitor the availability of the citric acid cycle intermediates [73].

Animal studies with selenocystine, ChSCA, and BSCA regarding their ability to decrease the size of existing tumors have not yet been conducted. Studies using xenograft models where animals are treated with these selenocompounds in combination with auranofin or other thioredoxin reductase inhibitors would provide evidence that these compounds can decrease tumor size *in vivo* and would also determine whether a decrease in tumor size with these compounds is enhanced with TR knockdown.

Finally, the ability of selenocompounds to synergize with current therapies such as cisplatin has not been evaluated. Selenocompounds have been shown to increase the

cytotoxicity and tolerated dose of anti-cancer drugs, including cisplatin [74, 75]. Cisplatin is an ideal anti-cancer drug to use in combination with selenocystine, ChSCA, or BSCA in the lung for several reasons. First, it is a common first-line therapy for lung cancer treatment and has established use in combination therapy for lung cancer. Second, cisplatin is known to inhibit thioredoxin reductase [76]. This would be an optimal feature of a drug to use in combination with selenocystine, ChSCA and BSCA, since their cytotoxicity is enhanced with thioredoxin reductase attenuation. Third, cisplatin resistance has been associated with high glutathione levels, as glutathione is involved in the detoxification and inactivation of platinum drugs [77, 78]. The ability of selenocystine, ChSCA, and BSCA to deplete glutathione in a thioredoxin reductase-independent manner may be especially useful in lung cancers that are cisplatin resistant [79]. Cell death may also be further enhanced in combination therapies with cisplatin or other anti-cancer drugs through genetic or pharmacological methods of TR1 attenuation. The ability of MSA to generate ER stress in lung cancer cells may also be useful in combination therapy. Several compounds have been shown to increase cisplatin efficacy through their ability to increase ER stress [80, 81]; similar effects may occur with MSA. The selenocompounds should also be investigated in the lung with other anti-cancer drugs that inhibit thioredoxin reductase, such as alkylating agents [82], or anti-cancer drugs that have increased efficacy and decreased toxicity with selenium, such as paclitaxel, etoposide, and irinotecan [83-85].

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APPENDIX A

5-OXOPROLINASE EXPRESSION AND ACTIVITY IN HUMAN LUNG CELL LINES

5-oxoprolinase (OPLAH) is the enzyme hypothesized to be responsible for the release of selenocystine from OSCA. This enzyme catalyzes the analogous ATP-dependent conversion of 5-oxo-proline to glutamate. As shown in Figure 2.1 in Chapter 2, the BEAS-2B cells were more sensitive to OSCA than the A549 cells. In fact, no change in viability was seen for the A549 cells at the highest concentration evaluated, 600 μM. However, selenocystine, the compound thought to be released from OSCA by OPLAH, did demonstrate toxicity in both cell lines. This differential toxicity of OSCA and selenocystine in the A549 cells, but not the BEAS-2B cells, led to the investigation of OPLAH. Studies of human tissues have shown lower OPLAH expression and activity in tumor tissue compared to adjacent, matched nonmalignant normal tissue, including lung [1, 2]. These previous studies and the differential effects of OSCA we observed in regards to viability led to the hypothesis that malignant A549 cells have lower OPLAH expression and activity than nonmalignant BEAS-2B cells. OPLAH mRNA expression and activity were assessed to determine if the differential toxicity of OSCA observed in

malignant and nonmalignant cells is due to differential expression or activity of this enzyme. OPLAH and mRNA expression was the same in malignant A549 cells as in nonmalignant BEAS-2B and NHBE cells (Figure A.1). In contrast with findings of previous studies, OPLAH activity was found to be the same in malignant cells as nonmalignant cells (Figure A.2). The similar OPLAH activity levels in the two cell lines may relate back to the similar OPLAH mRNA expression levels. These data indicate that the toxicity of OSCA in BEAS-2B cells that was not observed in A549s is not due to lower OPLAH expression or activity in the A549 cells.

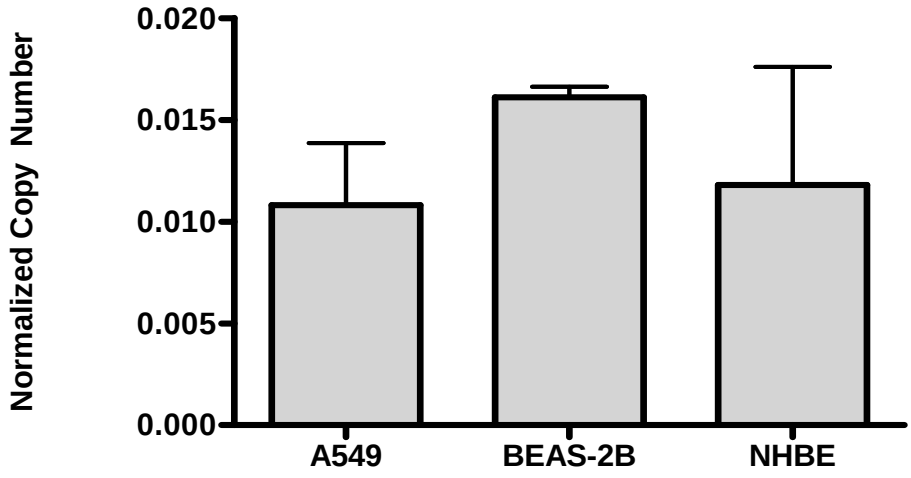

Figure A.1. mRNA expression of OPLAH in human lung cell lines. mRNA expression was quantified by QPCR and OPLAH copy numbers were normalized to PSMB6 copy numbers. Statistical analysis was performed using one-way ANOVA with Tukey post-hoc testing. No significant difference between groups was found. Data are presented as mean ± standard deviation. n = 3 for all groups.

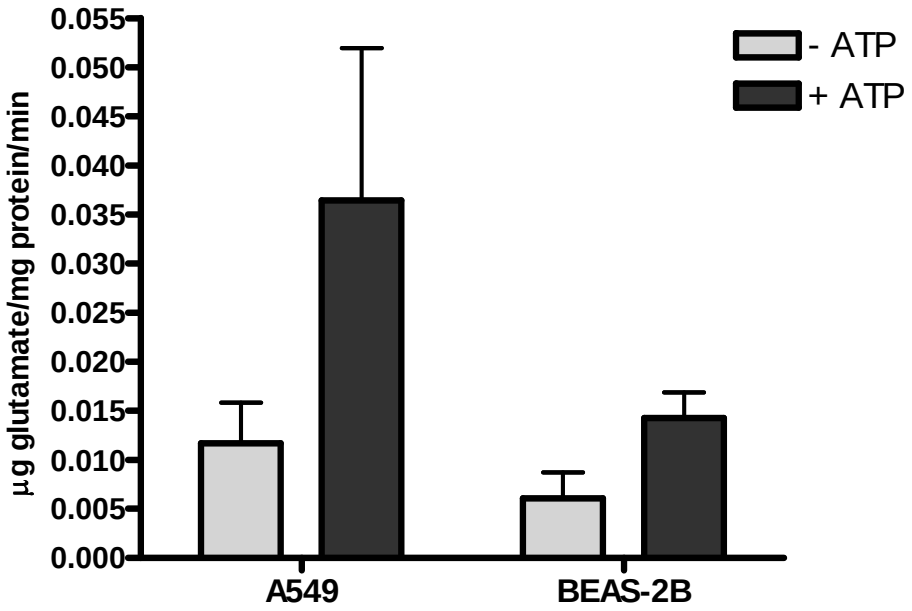

Figure A.2. OPLAH activity in human lung cell lines. Activity was determined by measuring the production of glutamate by whole cell lysates in a buffer of 50 mM Tris HCl, 0.1 mM EDTA, 5mM 5-oxo-L-proline, and 2 mM DTT [3]. This reaction was performed in the presence or absence of ATP for 45 minutes. As the reaction requires ATP, the reaction in the absence of ATP represents background glutamate levels. Measurements were made using a fluorimetric HPLC assay [3,4]. Relative fluorescence units were converted to µg glutamate using a glutamate standard curve. Data are presented as mean mg glutamate/mg protein/min ± standard deviation. n = 2 or 3 for all groups. Statistical analysis was performed on the normalized µg glutamate/µg protein/min ratio (+ ATP/- ATP) using one-way ANOVA with Tukey post-hoc testing. The ratios for A549s and BEAS-2Bs were 3.11 ± 1.32 and 2.35 ± 0.43, respectively. No significant difference between groups was found.

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APPENDIX B

MODULATION OF H1666 AND HOP92 HUMAN LUNG CANCER CELL REDOX STATUS BY SELENOCOMPOUNDS

The findings from Chapters 2, 3, and 4 indicated that the basal redox status and KEAP1 mutation status of human lung cell lines may be important determinates of the response of lung cells to selenocompounds. To further investigate the role of the KEAP1 mutation status, the H1666 human non-small cell lung carcinoma cell line was utilized. H1666 was shown not to have a KEAP1 mutation or loss of heterozygosity [1]. The HOP92 cell line was also assessed as another non-small cell lung cancer line. It is a large cell lung cancer cell line that has been shown to have a high tissue similarity index to lung tumor, indicating similar genetic characteristics [2]. One drawback of this cell line is that it is poorly characterized. It is presently unknown whether this line harbors a KEAP1 or NRF2 mutation that would lead to constitutive NRF2 activity similarly to A549 cells.

Free thiol and ROS levels were determined using mBBr and DCF, respectively, in cells treated with DMSO vehicle control to assess the basal cellular redox state. These measurements, along with KEAP1 status, are summarized for A549, BEAS-2B, H1666, and HOP92 cells in Table B.1. The A549 cells had a low free thiol level and the lowest ROS levels of the cell lines assessed. The BEAS-2B cells also had low free thiol levels, but the highest level of ROS levels assessed. As discussed in Chapter 2, these data

Table B.1. KEAP1 mutation status and basal mBBr and DCF fluorescence concentrations of human lung cell lines.

Cell Line	KEAP1 Mutation?[a]	mBBr (fluorescence conc.)	DCF (fluorescence conc.)
A549 (adenocarcinoma)	Yes	0.27 ± 0.04	0.009 ± 0.002
BEAS-2B (bronchial epithelial, virally transformed)	No	0.20 ± 0.05	1.40 ± 0.41
H1666 (adenocarcinoma)	No	1.40 ± 0.09	0.06 ± 0.01
HOP92 (large cell lung cancer)	Unknown	1.26 ± 0.15	0.43 ± 0.04

Cells were treated with 1 μL/mL DMSO as vehicle control for 24 hours. mBBr and DCF fluorescence concentrations were determined cytometrically as measure of cellular free thiols and reactive oxygen species, respectively, as described in Chapter 2. Data are presented as mean ± standard deviation. n = 2 or 3 for all groups.

[a], Reference 1.

indicated that the A549 cells were in a more reduced basal state than the BEAS-2B cells. The H1666 and HOP92s did not exhibit a basal redox state that totally mirrored either the A549 or BEAS-2B cell line. Similar to the A549 cells, basal levels of ROS were low in these cell lines, with neither cell line approaching the DCF fluorescence concentration of 1.40 seen with the BEAS-2B cells. In contrast to both the A549 and BEAS-2B cells, the H1666 and HOP92 cells had much greater levels of free thiols.

These data also demonstrate that KEAP1 status is not a good indicator of cellular redox state. This is evidenced by the H1666 line having higher free thiol levels and lower ROS levels than the BEAS-2B line, despite having the same KEAP1 status as the BEAS-2B line. Other pathways related to redox status may be modulated in this adenocarcinoma cell line compared to the nonmalignant BEAS-2B cell line.

Both the H1666 and HOP92 cell lines were treated with selenocompounds to assess the effects on cellular viability and redox parameters. At 48 hours, the H1666 cells shown IC_{50} values of approximately 5 μM for MSA and 10 μM for selenocystine and ChSCA (Figure B.1). BSCA had the lowest toxicity of the compounds assessed, with only a 25% loss in viability at 200 μM. The viability profile with selenocystine and ChSCA was almost identical as that for the A549 cells as shown in Chapter 4. MSA was more toxic and BSCA less toxic in the H1666 line than the A549 line. (Figure B.1). At24 hours, none of the compounds altered free thiol levels in H1666 cells (Figure B.2). This is in contrast to the ability of MSA, selenocystine, and ChSCA to modulate free thiol levels in both the A549 and BEAS-2B lines. The selenocompounds decreased ROS levels in the H1666s at 24 hours (Figure B.2), also in contrast to the selenocystine-mediated increase in A549s and lack of effect of the compounds on ROS levels in BEAS-2Bs.

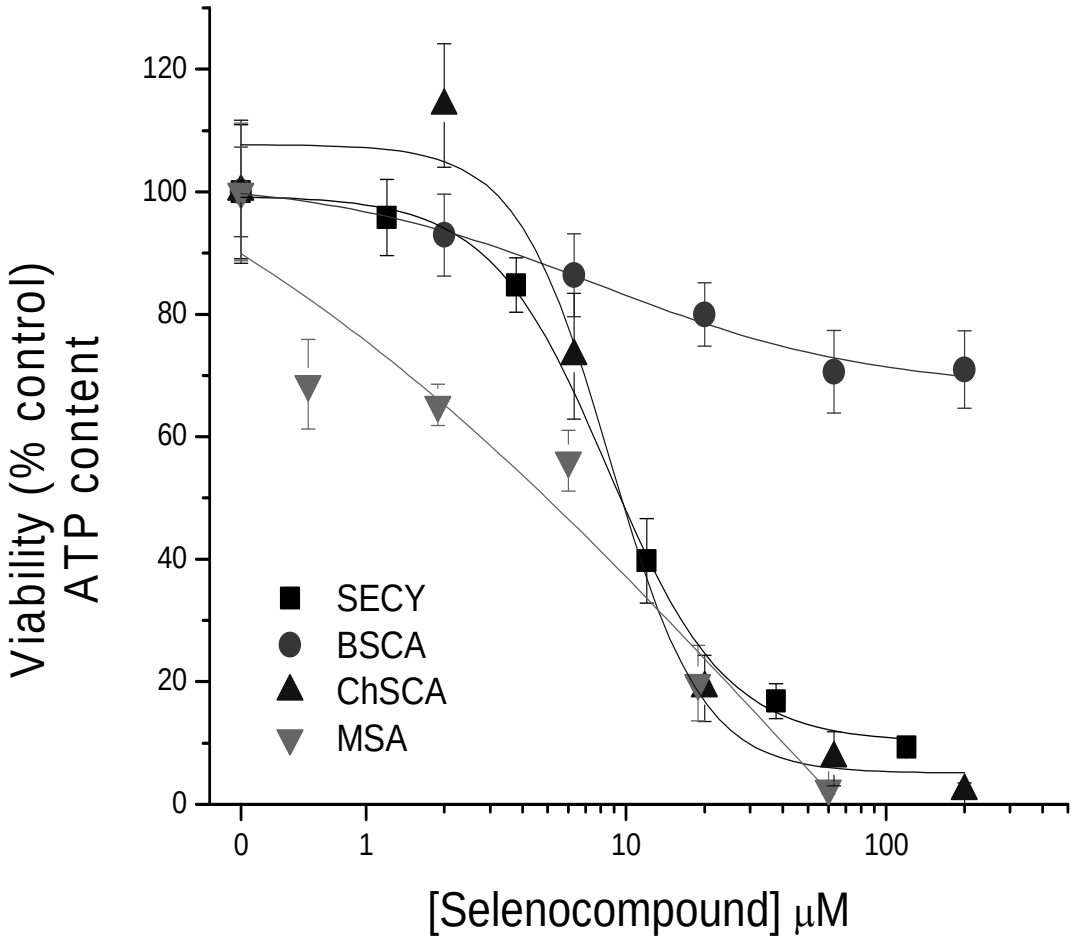

Figure B.1. Viability of H1666 cells following 48 hr selenocompound treatments. Viability was assessed by measuring ATP content. Data are presented as % control mean viability ± standard deviation. n = 4 for all data points.

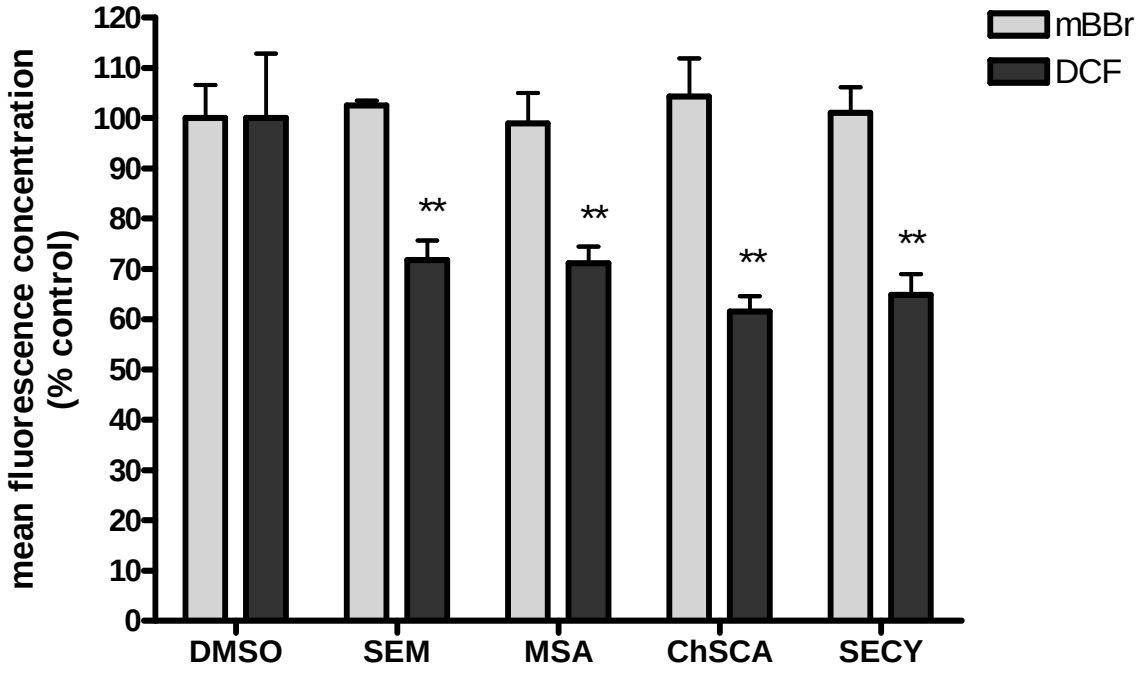

Figure B.2. Free thiol and ROS levels in H1666 cells following 24 hr treatments. Free thiols and ROS levels were assessed by measuring mBBr and DCF fluorescence concentrations cytometrically as described in Chapter 2. Treatment concentrations were 20 μM SEM, 2.5 μM MSA, 5 μM SECY, 5 μM ChSCA. Data are presented as mean viability ± standard deviation. n = 3 for all data points. One-way ANOVA with Dunnett's post-hoc testing was performed to determine statistical significant. No change in mBBr fluorescence concentration was found with any of the treatments. '**' indicates $p<0.01$ versus DMSO control.

At 24 hours, the HOP92 cell line demonstrates similar sensitivity to the selenocompounds as the A549 cell line (Figure B.3). However, the redox response of the HOP92 cells to was more similar to the BEAS-2Bs. The ability of the selenocompounds to modulation free thiol and ROS levels followed the same pattern in the HOP92s as the BEAS-2Bs, with selenocystine and ChSCA decreasing the free thiol content and none of the compounds altering ROS levels (Figure B.4). As with the A549s and BEAS-2Bs, selenocystine and ChSCA shifted the mitochondrial membrane potential of the HOP92s to the depolarized state (Figure B.5). The basal mitochondrial state was more depolarized than for the HOP92s than the other two cell lines.

In summary, the H1666 and HOP92 non-small cell lung cancer lines did not demonstrate similarities in keeping with either the A549 adenocarcinoma line or BEAS-2B nonmalignant line. Since a direct relation to either the line could not be made, even when taking into account the KEAP1 mutation status of the H1666 cells, the data for the HOP92s and H1666s was not included in Chapter 2. Overall, selenocystine and ChSCA tend to decrease free thiol levels and depolarize the mitochondria in human lung cells, with selenomethionine and MSA demonstrating little effects on these parameters. The H1666 cell line is the exception to these observations.

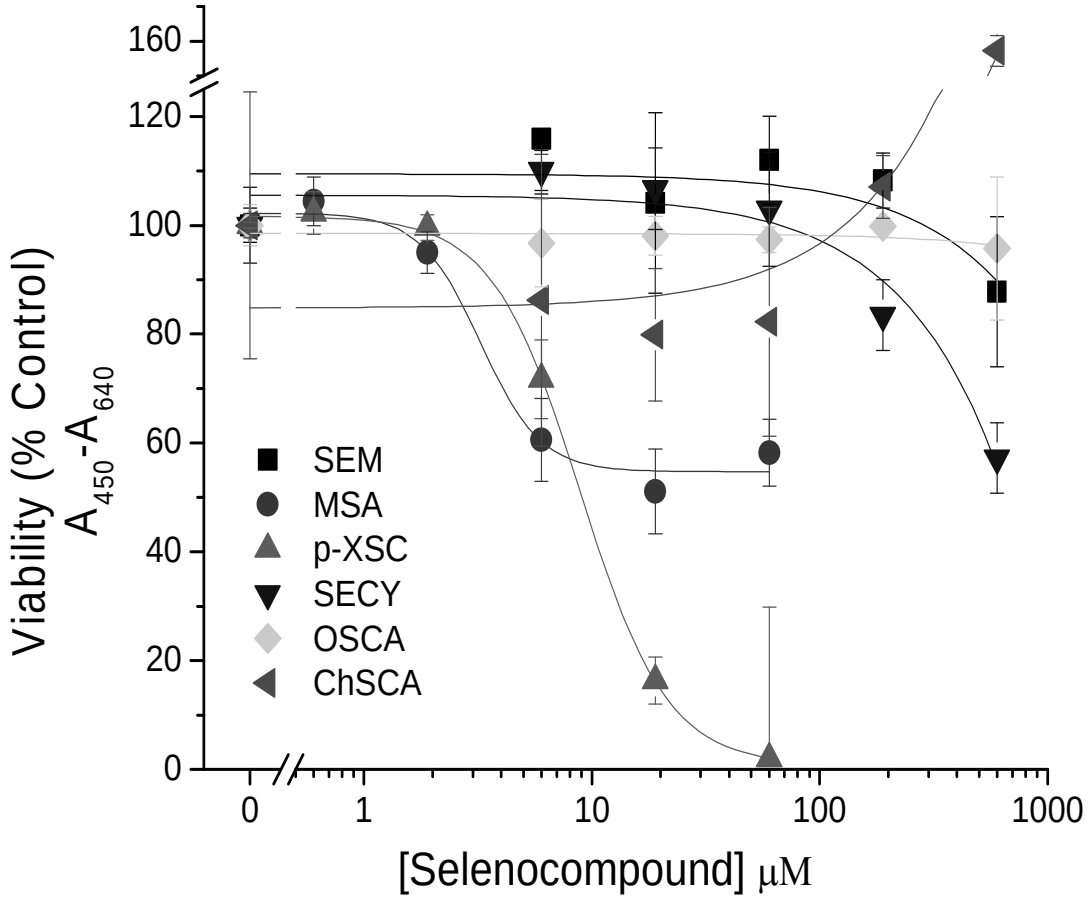

Figure B.3. Viability of HOP92 cells following 24 hr selenocompound treatments. Viability was assessed using tetrazolium salt reduction by NADH (CCK-8) as described in Chapter 2. Data are presented as % control mean viability ± standard deviation. n = 3 for all data points.

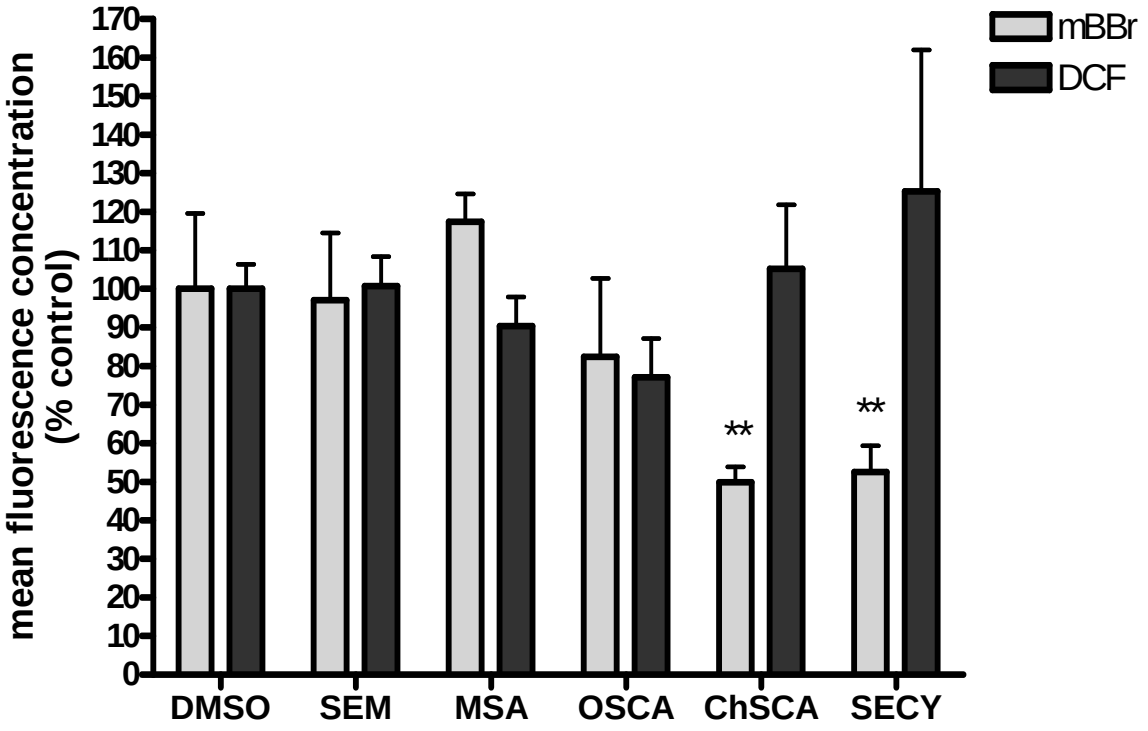

Figure B.4. Free thiol and ROS levels in HOP92 cells following 24 hr treatments. Free thiols and ROS levels were cytometrically measured using mBBr and DCF as described in Chapter 2. Treatment concentrations were 100 μM SEM, OSCA, ChSCA, and SECY and 2.5 μM MSA. Data are presented as mean fluorescence concentrations ± standard deviation. n = 3 for all data points. One-way ANOVA with Dunnett's post-hoc testing was performed to determine statistical significant. No change in DCF fluorescence concentration was found with any of the treatments. '**' indicates $p<0.01$ versus DMSO control.

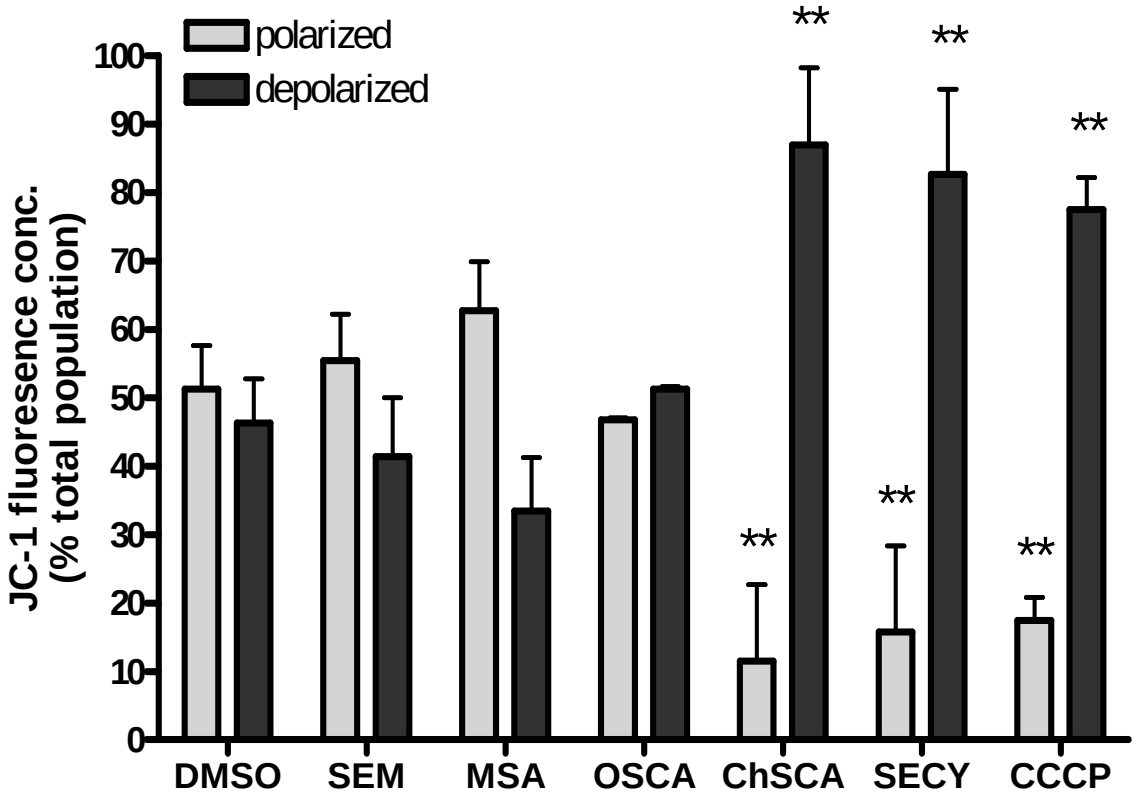

Figure B.5. Assessment of mitochondrial membrane potential in HOP92 cells following 24 hr treatments. JC-1 was used to determine polarized mitochondria (575 nm fluorescence; JC-1 red) and depolarized mitochondria (525 nm fluorescence; JC-1 green) populations cytometrically as described in Chapter 2. Treatment concentrations were 100 μM SEM, OSCA, ChSCA, and SECY, 2.5 μM MSA, and 25 μM CCCP. Data are presented as mean ± standard deviation. n = 3 for all data points. One-way ANOVA with Dunnett's post-hoc testing was performed to determine statistical significant. "**' indicates $p<0.01$ versus DMSO control.

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